Studies investigating the distinct effects of estradiol deficiency and aging on the redox system of skeletal muscle as it relates to strength and power

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Dedication

This dissertation is dedicated to my mother, who always believed in me and pushed me to follow my dreams.

Abstract

Aging results in a loss of skeletal muscle strength. This decline is further augmented by low levels of ovarian hormones in females, such as experienced following menopause. Power is arguably more critical to muscle function than is maximal strength, yet the impact of and mechanisms whereby aging and hormones impact muscle power are largely unknown. The studies of this dissertation utilize mouse models to investigate the impact of aging and removal of ovarian hormones on skeletal muscle maximal power and submaximal strength. The results of these studies are the first to convincingly demonstrate that ovarian hormones provide similar input to skeletal muscle function during adulthood and aging in females. This work lends further evidence for a critical transition in skeletal muscle function between 24 and 28 months of age in mice. Additionally, these dissertation studies reveal impacts of both aging and ovarian hormones on redox balance (oxidative stress levels and antioxidant system response) in skeletal muscle. Oxidative damage to contractile proteins of skeletal muscle increases with aging, and removal of ovarian hormones has a similar effect. It is speculated the increase in damage is a result of the decline in protein expression of key antioxidants, such as superoxide dismutase and glutathione peroxidase, that occurs with aging and when ovarian hormones are absent. Taken together, the results of this dissertation are suggestive of an important role of ovarian hormones in maintaining redox balance in skeletal muscle, particularly during aging in females. Further studies investigating methods to mitigate age-related changes are warranted.

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Introduction:

The loss of skeletal muscle strength experienced with aging is detrimental to one's ability to function and carry out tasks of daily living. Injury resulting from falls is one of the leading causes of hospitalizations in the elderly, with a noticeably greater incidence of falls and hospitalizations in women than in men [1]. Increased falls and resulting injury is associated with declines in muscle strength [1] , which leads to diminished independence and increased incidence of co-morbidities. Women and men experience different rates of skeletal muscle loss with aging [2]. This sex difference is the greatest at the time of menopause, and has been associated with the concurrent loss of estrogen [3]. Despite this knowledge that there are sex differences in the way that men and women age, the majority of studies in humans and rodents aimed at uncovering the mechanisms of age-related strength loss have included only males. This male-biased research focus on aging in skeletal muscle is a major limitation of current studies and results in a gap in understanding of the mechanisms through which estrogen deficiency detrimentally affects skeletal muscle in females and contributes to loss of strength.

While isometric force generation is a component of muscle function, dynamic contractions (including force generation at velocity and power) are arguably better predictors of the muscles ability to perform. Previous work by our laboratory has demonstrated that the impact of aging on muscle strength was muscle-fiber-type dependent. Aging impacted muscle components related to speed of contraction, even when muscle force generation was not impacted [4]. Studies in elderly humans have estimated that velocity and muscle power are stronger predictors of muscle function compared with maximal force or muscle cross sectional area [5-13] . These findings highlight the importance of velocity when investigating the impact of aging on muscle strength *in vivo*.

Previous work in rodents demonstrates that *ex vivo* muscle force generation in adult mice is lower when estradiol is lacking [14], which correlates with a decline in strong binding

myosin during contractions [15] and a decrease in phosphorylation of the regulatory light chain of myosin [16]. These beneficial effects of estradiol on myosin and muscle force generation appear to be altered with aging [17]. Thus mechanisms of estradiol's actions, especially as they relate to aging, require further investigation. A possible contributing mechanism to changes observed with estradiol deficiency is through alteration of the redox balance of skeletal muscle. Previous studies have shown that estradiol can affect oxidative damage caused by free radicals [18-24] and furthermore that these oxidative modifications accumulate with aging [25-36]. No studies have fully elucidated the pathway(s) through which estrogenic effects on redox balance and maintenance of force generation may occur, although there is a growing body of evidence for an ERα mediated mechanism [37-39].

Therefore, the primary goals of this dissertation are to 1) determine the extent to which and mechanism(s) whereby estradiol deficiency alters muscle strength and power in adult and aging female mice; 2) elucidate the impact of estradiol deficiency and aging on oxidative stress and damage to skeletal muscle and; 3) measure changes in antioxidant availability that may impair the redox-imbalance in aged and estradiol deficient skeletal muscle. Multiple studies have been conducted in order to test the over-arching hypothesis that estradiol deficiency is detrimental to many aspects of skeletal muscle contractile function.

Chapter #1: Literature Review

1.1 Age-related loss of muscle mass

Skeletal muscle mass is lost at a rate of approximately 1% per year past the age of 50 years [2]. The decrease in mass is attributed to a loss of both type 1 and type 2 fibers [40-43]. This loss of fibers translates to a loss of force production and muscle power, the latter of which is thought to be the major contributing factor to impair performance of daily living tasks (e.g., rising from a chair or walking stairs) [44, 45]. This decline in skeletal muscle mass and force production is termed sarcopenia and is associated with an increase in co-morbidity in aging [46]. The loss of muscle mass is due in part to loss of motor unit innervation, resulting in larger motor units as some denervated fibers are "adopted" by functioning motor units [1].

Although other motor units adopt many of the denervated fibers, yielding large motor units, a portion of these denervated fibers are not adopted. Without innervation these fibers will die, contributing to the sarcopenia witnessed with aging. There is some disagreement as to whether fast-twitch (type 2) fibers are lost preferentially over slow-twitch (type 1) fibers [47]. Previously it was accepted that fast-twitch were lost with aging whereas slow-twitch fibers were preserved. However, it has been proposed that this in fact is not the case; both fiber types are lost at a similar rate with aging [42, 47] and the number of fibers co-expressing as both fast and slow twitch (i.e., mixed fibers) increases with age [41, 48]. The increase in mixed fibers has consequences for not only force production of muscles but also the types of tasks that the muscles are able to perform. Thus in addition to the increasing size of the motor unit that occurs with denervation and re-innervation, there is a decrease in fast-twitch motor units as these become mixed fiber motor units [49, 50].

Another contributing factor to sarcopenia is muscle atrophy. Although muscle atrophy results from the loss of fibers, as previously discussed, decreased size of the remaining

muscle fibers is also observed with aging [51, 52]. Cross sectional area appears to be less affected by aging in type 1 fibers than in type 2 fibers where up to a 50% decrease is observed [46]. This loss of fiber number and size leads to a decline in whole tissue mass and force production [53, 54]. Muscle mass has been shown to decline by as much as 40% in humans from age 20-80 years [51] with a similar decline in the cross sectional area and the force producing capacity of the muscle [55]. Similarly, male and female rodents lose muscle mass and fiber size with age [40, 52, 56].

1.2 Age-related loss of force generation, velocity of contraction, and power

Loss of force generation outpaces loss of muscle mass with aging [57] indicating other contributing factors to muscle force production. In addition to loss of muscle mass, loss of force can be attributed to changes in the quality of muscle [58, 59], specifically by altering the contractile proteins (e.g., myosin). Myosin is the main contractile protein that makes up the thick filament in skeletal muscle's contractile apparatus. The interaction of myosin heads with the thin filament, in a strong binding state, causes force to be generated at the molecular level during a contraction. With aging there is a decrease in the strong binding of myosin during muscle contraction, resulting in reduced force production of individual fibers [60-62]. There is further evidence that the rate of myosin interaction with the thin filament is also decreased with aging [63]. The decline in strong bound myosin and rate of myosin binding [64], compounded with a decrease in the quantity of myosin protein [65-67], plays a role in the loss of skeletal muscle force generation that occurs in aging.

In addition to changes in myosin, it has also been proposed that alterations in the neuromuscular junction (NMJ) diminish force signal transmission [68]. Specifically, it has been suggested that disorganization of the synaptic cleft leads to disassembly of the NMJ which likely contributes to the aforementioned alterations [69, 70]. Proper functioning of agrin, a signaling factor for the formation of the NMJ, is necessary to maintain acetylcholine release and structural organization [69]. Cleavage of agrin results in a sarcopenia phenotype [70] similar to that observed with aging, lending further support to the importance of a properly organized NMJ in maintenance of skeletal muscle mass and function.

Combined, these age-related changes result in loss of force production that is observed in both men and women, with the most noticeable loss occurring at age 50 years and greater at which point a rapid decline in force production occurs in women, coinciding with

menopause [2]. Due to the high inter-individual variability associated with aging, the cross-sectional study design employed by the majority of studies may tend to underestimate the decline in muscle size and force production. Unfortunately, longitudinal studies that better identify the age-related alterations that occur in skeletal muscle [51], present far greater challenges in implementation and are less frequently employed. Another important note about current studies investigating the age-related changes in skeletal muscle, is that the majority of these studies have focused solely on males [46, 51]. Differing physiological changes that occur during aging between males and females suggest that the mechanisms behind aging-associated skeletal muscle force loss are also different between sexes [71, 72]. One of the primary focuses of this dissertation is to contribute additional studies focused on females to better delineate female-specific changes with aging.

Another functional change that occurs with aging is a decrement in contractile velocity of muscle movement. Power, the ability to produce force at a specific velocity, is arguably more critical to study than isometric strength as people experience a 3.5% per year rate of decline in power compared to the declines of 1.5% per year in muscle strength [73]. Additionally, power is a more comprehensive measurement of muscle function and changes to the muscular system than isometric strength and is a better predictor of muscle overall function [74-77]. However, the majority of studies that investigate changes in muscle with aging focus on maximal contraction strength; relatively few studies explore age-related changes in velocity and power.

As with much muscle aging research, investigations into alterations in muscle power in humans have focused on men. From the few studies that have considered sex differences, it is clear that muscle power decline with aging in women is different than what is observed in men. The results of studies carried out to date suggest an age-related decline in muscle power, specific to changes in the peripheral nervous system and intrinsic factors of the muscle. A decline in power can also be due to atrophy (i.e., less motor units); however, the majority of studies attribute power loss to a decline in torque

production and velocity with minimal impact of muscle size [77, 78]. Furthermore, it was suggested that the decline in velocity is due to slower development of twitch contraction [78]. It is evident that muscle power is impacted by a multitude of factors [79, 80], of which the contributions at a cellular level are not yet defined [73]. If we are to improve muscle power maintenance with aging, it is imperative that cellular mechanisms be understood.

Investigations utilizing rodent models, such as those performed in this dissertation, are necessary in order to isolate factors that contribute to strength and power, as manipulations of hormones, proteins, etcetera are easily performed. Only by isolating pathways can we identify the mechanisms contributing to changes in muscle strength and power with aging. Several studies to date have sought to identify changes in power with aging in mice. Collectively these studies confirm an age-related decline in muscle power that is exacerbated when the muscle is required to contract at near maximal force output [56, 81-83]. These studies fail to fully address the contribution of velocity to power production and results are limiting as these studies used only male rodents and the muscles were tested *ex vivo* [83]. A previous study performed in our lab found evidence for a slower rate of contraction and relaxation in muscles from female mice in muscles *ex vivo* [4], suggestive of alterations in velocity with aging. Thus, a main focus of this dissertation is to investigate the effect of age on muscle power production across a wide range of velocities in female mice.

1.3 Estrogens and estrogen receptors

Estrogen, the main female sex hormone, is a polyphenol biomolecule produced mainly by the ovaries, although there is also limited production by other tissues (i.e., adipose tissue). Estrogen naturally occurs in three forms: estrone (E1), estradiol (E2), and estriol (E3), numbered thus based on the number of hydroxyl groups present. Estradiol (E2) is the most bioavailable form of estrogen in the body and is found as 17α-estradiol and 17β-estradiol [84]. Plasma concentrations of 17β-estradiol in premenopausal women are ~0.2–1.0 nmol/L; however, these levels can be considerably higher in various tissues [84].

Many of estrogen's functions are mediated through the estrogen receptor (ER). Three types of estrogen receptors have been identified, ER alpha, ER beta, and G-protein coupled ER (GPER) [85-87]. Estrogen receptors alpha and beta primarily localize to the nucleus to have their effects, although a small percentage can be found in the cytoplasm [88]. The main ligand for ERs, 17β -estradiol, is hydrophobic which allows it to diffuse through membranes into the cells where it can bind the receptor [89]. ERs have been shown to play a role in gene expression (genomic effect) and cell signaling (non-genomic effect) [90].

The genomic effects have been largely attributed to be due to ERs alpha and beta, whereas the immediate non-genomic effects are hypothesized to occur mainly through the membrane-bound GPER [84, 91-94]. There is evidence for tissue-localization of specific receptor types [90]. Estrogens have been shown to have widespread effects in many body systems (i.e., nervous system, cardiovascular system, reproductive system, immune system, and musculoskeletal system); however, how estrogens function in these systems and to what extent the observed effects are through genomic and non-genomic actions is highly tissue specific and new details are continually being elucidated. Because estradiol is the most bio-available form of estrogen, it is the hormone of interest for these dissertation studies.

1.4 Age-induced decline in estradiol production

In women estradiol levels fluctuate based on menstrual cycle phase from the time of puberty up until the time of menopause. The National Institute on Aging and others define menopause as 12 consecutive months without menstruation. At the time of the menopausal transition, which averages age 51 in women, there are alterations in the levels of several hormones, the most notable of which is the drastic decline in estradiol [95]. Decline in ovarian follicle number below a critical threshold is considered to be the main driving force in menopause, ultimately resulting in ovarian failure [96]. Since the ovaries are the primary estradiol producing tissue in the body, ovarian failure causes a sharp decline in estradiol levels. The low levels of estradiol adversely affect various body functions including bone turnover, lipid levels, and skeletal muscle function [97, 98].

A similar cycle of fluctuating estradiol levels is observed in mice. This is termed the estrous cycle [99, 100] and it begins at the time of sexual maturity (approximately 2-3 months of age) continuing until ovarian failure/ senescence, at 18-20 months of age [17, 100]. The estrous cycle is similar to the menstrual cycle in that hormone (estrogens, follicle stimulating hormone, progesterone, and inhibin) levels fluctuate in a regular, cyclic manner within a physiological range and changes in these levels of hormones determines fertility. Similar to menopause in women, declining levels of estradiol in aging female mice result in ovarian senescence [100].

The ovariectomy mouse model is commonly used as an induced menopause model in adult female mice [101-103]. Although this approach results in an abrupt decline in estradiol levels as opposed to the more gradual decline observed in menopause, it is a useful tool to study the effects of estradiol depletion without confounding effects associated with aging. An approach used within our lab to investigate the effects of estradiol treatment is to ovariectomize mice and replace the hormones lost by removal of the ovaries with 17-β estradiol [14, 15, 17], mimicking estrogen-based hormone

replacement therapy in women. This approach permits for the determination that estradiol is the ovarian hormone implicated, versus other ovarian hormones such as progesterone.

1.5 Effects of estrogens on skeletal muscle strength and power

Hormone therapy (estrogen-based and combined estrogen/progesterone treatments) has been shown to maintain muscle strength in post-menopausal women [104]. A growing body of evidence confirms the detrimental effects of estrogen loss on skeletal muscle. It has been shown that the loss of estradiol at menopause has a significant impact on muscle power in women. One study found that premenopausal women have 95% greater power than post-menopausal women [105] , and in post-menopausal women hormone therapy can partially protect against that loss. Some attempts at elucidating the mechanism of this decline when estradiol is low have been undertaken, results of which suggest a role in mitochondrial function [106]. However, as previously discussed, it can be challenging to investigate mechanisms in humans, and studies in rodents are necessary to probe cellular mechanisms.

Previous studies in rodents have shown that loss of estradiol results in decreased force generating capacity as well as reduced myosin function [98, 107, 108]. Furthermore, restoration of estradiol levels seems to recover muscle function [15]. This is further substantiated by a meta-analysis which supported estrogen-based hormone replacement therapy in post-menopausal women as well as estradiol treatment of female ovariectomized rodents to improve muscle force production [104]. The extent to which estrogen maintains muscle function remains controversial and the mechanisms by which this is accomplished are not fully understood [109] although it is plausible that this hormone affects redox balance (oxidant/antioxidant ratio) in skeletal muscle since it affects redox balance in other tissues (discussed in further detail in section 3). To date, no studies have been able to convincingly demonstrate a redox mechanism for the effects of estradiol on skeletal muscle.

To further delineate effects of estrogens and other sex hormones on muscle force production, a number of studies have investigated the correlation between menstrual cycle phase (high or low levels of estrogen and progesterone) and performance in

women. Studies investigating whole body submaximal and maximal performance (e.g., running, cycling, etc) found little to no effect of menstrual cycle phase on muscle performance [110-113]. Whereas studies investigating strength of a single muscle over the course of the menstrual cycle give evidence for estradiol-driven changes in maximal output [114]. Further, the role of female sex hormones on skeletal muscle performance is still unclear.

1.6 Effects of aging on oxidative stress and damage

The production of free radicals (ROS) at low levels is necessary for signaling, for example during gene regulation and the immune response [115, 116]. As such, limited, small amounts of ROS production are good and necessary for the body, but large amounts can be detrimental. The oxidative stress/redox theory of aging is a leading theory explaining many of the physiological changes that accompany aging in the body [117-119]. This theory of aging, introduced by Denham Harman in 1956 [120], postulates that the hallmarks of aging are due to an increased formation of free radicals resulting in oxidative stress and damage to tissues. It has been subsequently supported by numerous studies showing the accumulation of oxidative damage with aging in many tissues [32, 36, 65, 121, 122]. Despite decades of research on this topic, the collective results are mixed and some data indicate that oxidative damage with aging is tissue-dependent [27, 34, 36, 123, 124]. One key point consistent across studies is the importance of damage to cells, whether that damage is a result of oxidation overload or lack of mechanisms to combat oxidation, such as attenuation of antioxidant defenses [28, 29, 35, 119, 125, 126]. This is often referred to as an imbalance in the oxidant-antioxidant ratio and is important not only in aging but also in many disease processes. There are two possible ways to negatively alter this ratio: 1) through increased free radical generation and/or 2) through decreased antioxidant response [126].

Increased free radical generation by mitochondria is associated with aging [22, 127, 128]. At the same time, plasticity of the antioxidant response is reduced [129, 130], resulting in tissue that is less able to up-regulate antioxidants to combat an oxidative insult. The phenomenon of elevated oxidative stress in conjunction with lack of antioxidants has been well demonstrated in the aging brain [131-133] and is a major contributor to many age-related neurodegenerative diseases. Due to the loss of function that results from age-related diseases, the focus of aging research and the oxidative damage theory has shifted to identifying factors that contribute to promoting healthy aging (healthspan) rather than extending lifespan [32]. Healthspan can be equated to increasing the number of years that

normal physical activities are maintained. While longevity is important, the current public health perspective places greater importance on improving health in later years and reducing morbidity leading to mortality. The research focus of this dissertation, to improve skeletal muscle function by better balancing oxidative stress with age, aligns with this public health goal.

1.7 Oxidative stress and damage in skeletal muscle

Skeletal muscle is a highly metabolic tissue that produces and experiences high levels of oxidative stress [134, 135]. Mitochondria are a main source of ROS production in skeletal muscle, specifically the leak of superoxide from complexes one [136, 137] and three [138] in the electron transport chain [139-141]. However, NADPH oxidase (NOX) [135, 142, 143] and Xanthine Oxidase (XO) [144, 145] are additional sources of ROS production in muscle. Levels of oxidative stress fluctuate in skeletal muscle in relation to the levels of functional demand placed on the tissue. When muscle activity levels are high, such as that experienced during intense exercise, mitochondrial ATP production and therefore ROS production increase [146]. This has been demonstrated by numerous studies in adults [147, 148] and aged individuals [33, 149].

Levels of ROS and antioxidant production are altered with aging even under conditions of normal use (i.e., not intensive exercise) [150] and as previously discussed, aging is hypothesized to alter the antioxidant response. Thus, interpretation of the effects of aging on the oxidant and antioxidant balance specifically in skeletal muscle is difficult. As such, global markers can be useful in addressing changes in oxidation that occur. One commonly utilized global marker is biopterin (BP), formed from the oxidation of tetrahydrobiopterin (BH_4), an essential cofactor in the synthesis of a number of neurotransmitters including dopamine, norepinephrine, epinepherine, and serotonin. BH_4 also plays a crucial role in nitric oxide synthesis. BH_4 can be synthesized through both the *de novo* and salvage/recycling pathways of several tissues including brain, spleen, liver, kidney and adrenal glands. The *de novo* pathway requires the enzymatic conversion of guanosine triphosphate (GTP), resulting in the formation of BH_4. The salvage/recycling pathways utilize a series of enzymes that convert the by-products of BH_4 utilization back to BH_4 [151-156]. Biopterins cycle between dihydrobiopterin (BH_2) and BH_4 depending on oxidation state; however, under conditions of oxidative stress BH_2 can be non-enzymatically oxidized to BP providing an indication of oxidative status.

Additionally, BH_4 serves as the catalyst for the production of nitric oxide (NOS); the role of BH_4 in this enzymatic process is so critical that some research points to a deficiency of BH_4, and thus of nitric oxide, as being a core contributor to the neurovascular dysfunction that is the hallmark of circulation-related diseases such as diabetes. Recent research into the role of BH_4 in muscle function has provided some evidence to support that the decline in vasodilation of muscles that occurs with aging is in part due to limited BH_4 availability [157-159] which in turn results in decreased NOS [160] and reduced exercise capacity [161]. Furthermore, it is suggested that reduced BH4 availability can result in uncoupling of NOS resulting in increased production super oxide [162].

In addition to limitations in BH_4 availability that occur with aging, it is hypothesized that there is an increase in irreversible oxidation of BH2 to biopterin (BP), due in part to increased ROS generation resulting from dis-regulation of NOS [163]. Specifically, low levels of BH4 are credited with uncoupling of eNOS which drives the system to produce superoxide radicals instead of nitric oxide [162, 164, 165]. Once BH4 is oxidized to BP, it is unable to participate in regulation of NOS activation. Thus, further reduction of the bioavailability of BH_4 through this mechanism will drive the system to produce more free radicals (Figure 1).

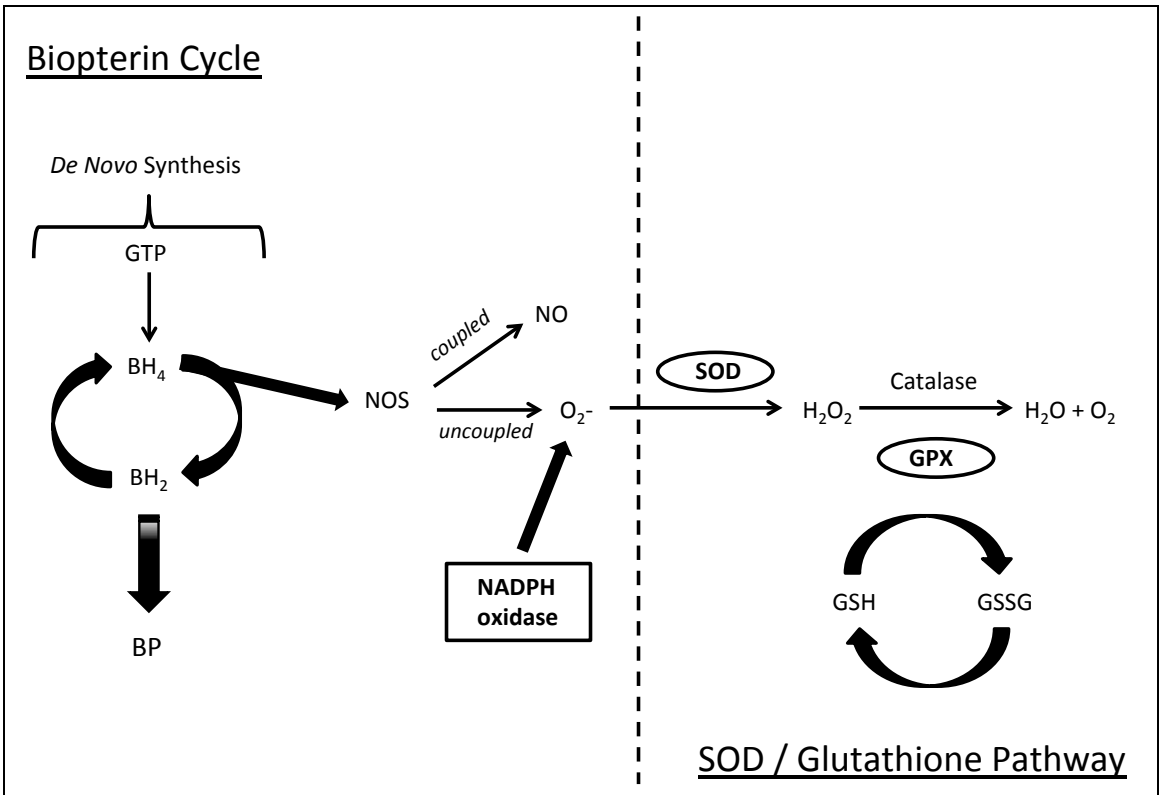

Figure 1: Overview of the formation of biopterin and its relationship with reactive oxygen species generation and the endogenous antioxidant system of superoxide dismutase and glutathione.

1.8 Effects of estrogens on oxidative stress and damage

Sex differences in oxidant production by mitochondria have been shown, and it is hypothesized that these differences are due to effects of estrogens [166-168]. Recent research supports a critical role for estradiol in managing energy metabolism of the mitochondria and overall mitochondrial health [38, 39, 106, 169]. Furthermore, cell susceptibility to stress [170] and damage to DNA from oxidative stress are higher in males than females [171]. There is some evidence for the protective role of estrogens or estrogen-like compounds, against oxidative induced damage in muscle cells [18, 172-174], endothelial cells [175], and other cells of mesenchymal lineage [176-179]. Specifically, estradiol has been shown to be protective against hydrogen peroxide-induced oxidative damage in cultured muscle cells, but not much is known about the effects of estradiol on muscle fibers and skeletal muscle tissue *in vivo* [18].

The mechanisms through which estradiol is protective to non-reproductive tissues and cells, as well as the extent to which this protection is afforded with aging, are not fully understood. As discussed previously, estradiol can act through estrogen receptors (ERs) in a genomic fashion as well as through a non-genomic fashion via membrane receptors and direct activation of other molecules. The current evidence indicates protection against oxidation via ER-activated responses in adipose tissue [23], smooth muscle [180], and cardiac muscle [181]. Involvement of ERs in estradiol signaling is further substantiated by evidence of ER utilization for skeletal muscle regeneration [182-184] and metabolism [185-187]. The hypothesis that estradiol is acting primarily through ERα, as opposed to ERβ, is based on work by a previous member of our laboratory who reported that ERα is the most abundant ER in mouse skeletal muscle, has higher expression in fast-twitch than slow-twitch muscle, and is sensitive to changes in circulating levels of estradiol [37]. Recently a study was carried out on skeletal muscle specific ERα knockout mice that further supported this mechanism for estradiol in skeletal muscle, particularly as it relates to metabolism. Diminished muscle oxidative metabolism accompanied by muscle

dysfunction was observed in mice lacking ERα [38] suggestive of a role for estradiol in oxidative metabolism of skeletal muscle.

1.9 Effects of aging on antioxidants

Changes in antioxidant levels with exercise and aging is a topic of considerable interest; however, most research has focused on intake of antioxidants such as Vitamin E or Vitamin C, and few have investigated the endogenous enzymatic antioxidants such as Glutathione or Superoxide Dismutase. As previously discussed, with aging the response of skeletal muscle to stress is blunted (i.e., muscle is less able to adapt to changing conditions with age). This is further evidenced by changes in the ability of skeletal muscle to adapt to exercise with aging. It is well known that exercise induces production of reactive oxygen species (ROS) and that healthy muscles adapt antioxidant defense systems to combat the increase in ROS. Specifically, exercise has been shown to up-regulate superoxide dismutase, catalase and glutathione peroxidase [188-190].

Early studies investigating endogenous antioxidants suggested a decline in antioxidants with aging [191-196]. More recently, studies investigating antioxidant levels in aged skeletal muscle have yielded conflicting results. For example, one study found no effect of aging on basal levels of antioxidants but when subjected to exercise, levels of glutathione and glutathione peroxidase increased [197] while another found decreased levels of glutathione content with aging and no response of the antioxidants to exercise [198] and in yet another study increases in manganese superoxide dismutase and catalase activity were observed concurrent with the age-induced increases in oxidative stress [31]. Studies focusing on the impact of aging on superoxide dismutase are easier to interpret. In male rodents, with aging there is a decline in expression of superoxide dismutase [199] these results are further corroborated by studies utilizing SOD1 knockout mice. These studies found that deletion of SOD1 in young mice resulted in a skeletal muscle contractile phenotype similar to that of aged mice [138, 200], suggesting that SOD1 is critical for the ability of muscle to adapt/neutralize reactive oxygen species following exercise [201, 202]. With aging, this adaptability in skeletal muscles following exercise is abolished [149, 203].

Collectively, literature is in agreement on the importance of superoxide dismutase, catalase, and glutathione peroxidase on the function of aged skeletal muscle, but it remains to be determined how these levels are impacted by exercise with aging. Thus, a goal of this dissertation is to investigate the impact of aging on antioxidant levels in skeletal muscle following exercise.

1.10 Effects of estrogens on antioxidants

A limited number of studies have investigated the impact of estrogens on antioxidants in skeletal muscle. Research in women during different phases of the menstrual cycle is contradictory with some studies reporting increased blood levels of antioxidants, specifically superoxide dismutases, following exercise when estrogen levels are high [204] and others reporting no impact of menstrual phase on antioxidants [205]. In cardiac muscle, estradiol deficiency results in low levels of glutathione peroxidase, catalase, and superoxide dismutase [206, 207]. Previous studies in female mouse skeletal muscle have found that regulation of several antioxidant genes is affected by estradiol [37]. Of particular interest were glutathione peroxidase 3 (Gpx3), glutathione Peroxidase 1 (Gpx1), and NADPH Oxidase 4 (NOX4) as these antioxidants were known to be sensitive to aging and estradiol treatment. Further studies performed in cultured muscle cells, demonstrated that treatment with estradiol increases concentrations and activity of SOD2 [208], although the mechanism was not investigated. Taken together, these studies are suggestive of a role for estradiol in regulating antioxidant availability.

The role of estradiol in regulation of antioxidants in skeletal muscle has been minimally investigated and the limited results warrant additional investigation to elucidate the effects of estradiol deprivation on not only oxidation in skeletal muscle, but also the mechanisms that underlay these changes (i.e., antioxidant regulation). The studies in this dissertation seek to further examine the effects of estradiol deprivation on antioxidants, and additionally, to determine if this effect is maintained or altered with aging.

Chapter #2: Impacts of estradiol and aging on sub-maximal strength and power

There is ample evidence for strength loss with aging; however, most studies have focused on males when investigating strength loss and the underlying mechanisms. Limited studies on strength loss with aging in women have demonstrated a beneficial effect of hormone-replacement therapy on maintaining muscle strength. But the variability in study design, time of therapy initiation, duration of therapy usage, and type of therapy employed make it difficult to come to consensus. Several studies have sought to investigate this further by utilizing rodent models, where estradiol levels and treatment can be better controlled. Most previous studies examining the effects of aging and hormones on female mouse muscle have utilized an *ex vivo* approach, with small hindlimb muscles, such as the soleus or extensor digitorus longus (EDL) muscle dissected from the animal. This approach has many advantages but is also limiting because a) it cannot accommodate larger muscles due to the limitation of oxygen diffusion to core fibers as the vasculature is disrupted when the muscle in removed from the animal b) the loss of other environmental factors delivered in the blood, c) contraction is initiated downstream of the neuromuscular junction so any involvement of that structure is not measured, and finally d) smaller muscles have less of a contribution to hindlimb strength and power so investigating larger, more functionally-relevant muscles and muscle groups is advantageous.

Thus, the following studies employ an *in vivo* approach to muscle function testing which allows for testing the muscle in a more functionally relevant manner in an anesthetized mouse. The muscle is left in the native environment and rather than testing a single muscle, this approach allows for testing of a muscle group in the same manner as these muscles would be elicited by the mouse during normal ambulation. Also limiting in previous studies was the use of only adult ovariectomized mice, and not aged mice that had undergone the same treatment. To fully address the effects of ovarian hormones, and specifically estradiol, both adult and aged mice must be subjected to the same treatment and timeline.

The first study of this dissertation utilizes adult and aged female mice to ask the following questions: Given what is known about the effects of estradiol deprivation on maximal strength of skeletal muscle, what is the impact of ovariectomy on the more physiologically relevant measures of sub-maximal muscle strength and muscle power? Can replacement of estradiol counteract the effects of ovariectomy? Do aged mice respond similarly to treatment as adult mice?

Chapter 2: Impact of aging and ovarian hormones on skeletal muscle function

Study #1 - Part A:

Hypotheses –

Ovariectomy will result in lower muscle power and submaximal torque generation in adult female mice compared to ovary-intact female mice, and treatment with estradiol will reverse these effects. Aged, ovarian senescent female mice will have lower muscle power and submaximal torque than adult mice. Ovariectomy in the aged, already ovarian hormone-deficient mice will not impact muscle contractility, but treatment with estradiol will return strength and power to levels observed in adult mice.

Study design –

Adult (4 month old, n=24) and aged (22 month old, n=28) female C57BL6 mice were obtained from the National Institute of Health Aging Colony. Vaginal cytology was performed for four consecutive days to confirm normal estrous cycling in adult mice and ovarian senescence in aged mice. Cellular markers of normal estrous cycling as well as those that accompany ovarian senescence are described in detail in the vaginal cytology methods section below. Adult and aged mice were randomly divided into three treatment groups: control, ovariectomy (OVX), and ovariectomy with 17β-estradiol treatment (OVX + E2). Mice in the control group were maintained in normal specific pathogen free (SPF) housing until time of testing and sacrifice. Mice in the OVX and OVX+E2 groups underwent bilateral ovariectomy as described in detail in the methods below. 17β-estradiol treatment in the OVX+E2 group was initiated at time of ovariectomy and was accomplished via a subcutaneous implanted time-release pellet, as described in detail in the methods below. Vaginal cytology was once again performed in the OVX group for 4 consecutive days to verify effectiveness of ovariectomy.

Approximately 3 months after surgery (and hormone treatment), mice were anesthetized and *in vivo* muscle function of the posterior crural muscles was assayed. Maximal

isometric torque, submaximal isometric torque (via a torque-frequency protocol), and power (via a torque-velocity protocol), were measured as described in detail in the methods below. Following *in vivo* testing, muscles were harvested and stored at -80°C for further biochemical analysis to address oxidative stress questions in a subsequent study. Uteri were weighed as secondary confirmation of effectiveness of ovariectomy.

Statistics –

Statistical power calculations were used to determine ideal sample sizes for studies necessary to apply an alpha level of 0.05 with 80% power. Expected variability was determined from previous studies performed in our laboratory and the wider literature base. Data were analyzed using a two-way ANOVA (age x ovarian status). Since there were no statistically significant interactions, all p-values reported are main effects of age and ovarian status. For all statistically significant main effects of ovarian status, a Holm-Sidak post-hoc test was implemented. All statistical calculations were performed on Sigma Stat statistical software.

Methods –

Often, adult mice are manipulated pharmacologically, genetically, or surgically to model aging. While those approaches can be useful tools and are often a necessity due to the difficulty in obtaining old mice and the time-consuming nature of aging the mice for a study, there are important limitations to consider and address. This is especially true when using young ovariectomized mice to study the effects of estradiol deprivation. The majority of studies that have been performed in attempt to elucidate the effects on skeletal muscle have deprived mice of hormones for 1-2 months. However, if we are truly attempting to investigate the effects of estradiol deprivation as it occurs in the context of aging, then the duration of deprivation becomes exceedingly important. Ovarian senescence occurs at 18-20 months of age in mice, though some reports suggest this may happen even earlier [14, 98-100, 209-213]. Therefore, by the time mice reach an age at which they are considered old (24 or 28+ months), they have been senescent for a minimum of 4-6 months. With this in mind, studies conducted in this dissertation using

adult hormone deprived mice have sought to investigate not only the effects of estradiol deprivation, but also the effects of duration of deprivation.

Vaginal Cytology

Vaginal cytology is important to track when studying females as estradiol and other ovarian-derived hormones can impact the outcome measures of a study if those measures are hormone sensitive. For this dissertation, cytology was an important tool that allowed mice to be studied at high and low levels of naturally circulating estradiol. Furthermore, it served to verify senescence of ovaries, when ovaries stop producing hormones similar to menopause in women, and also to confirm effectiveness of ovariectomy surgeries.

Accounting for estrous cycle is often an overlooked aspect of female studies, yet can be highly informative when interpreting results. The estrous cycle is driven by changes in circulating hormone levels and as a main component of this dissertation is to investigate effects of estradiol deprivation it was critical to confirm that young adult ovarian-intact mice had normally circulating levels of estradiol. The vast majority of studies utilizing female rodents fail to report tracking of estrous cycle; this can be easily accomplished and is a tool that should be utilized by all female rodent studies moving forward when estradiol is implicated. Furthermore, because of the changing hormone levels in female rodents, most studies of aging muscle have used male rodents, as they do not need to account for fluctuating or declining hormone levels. While it is true that many biological variables likely are not affected, evidence from several studies demonstrate that muscle contractility can be affected by estradiol deficiency, at least after the hormone has been low for 4-8 weeks [4, 15, 104].

Vaginal cytology swabs were taken as follows to identify the estrous cycle phase of mice. A phosphate buffered saline wetted cotton swab was inserted approximately 1-2 mm into the vagina and rotated 360 degrees to acquire cells. Cells were then transferred from the cotton swab to a statically-charged microscope slide and stained in hematoxylin. Slides were rinsed and then visualized under a microscope at 5-10X magnification. Changes in

ovarian hormones drive the alterations in cell population that are observed. In short, the lowest level of estradiol is present at the metestrus and diestrus phases whereas the highest level of estradiol is present at the proestrus and estrus phases [209, 214]. The following cell features were used to identify cycle phase [100, 209, 215, 216]:

> Diestrus – cells are predominately leukocytes; nucleus is clearly visible and occupies the majority of cellular space; often accompanied by mucus (Figure 2A).
> Proestrus – cells are transitioning to estrus; cells are predominately nucleated epithelial cells; nucleus remains clearly visible but additional cytosol of cell surrounding nucleus is apparent (Figure 2B).
> Estrus – cells are predominately cornified epithelial cells; nucleus is no longer apparent and cytosolic volume occupies the majority of the cellular space; cells are well formed (Figure 2C).
> Metestrus – cells are transitioning to diestrus; cells are predominately collapsing epithelial cells; cells lose structure and beginning to fold onto one another, nucleus may begin to become apparent (Figure 2D).

Following ovariectomy, when estradiol levels are low, estrous cycles will be absent and cytology will become consistent (Figure 2E). Similar lack of cycling will occur at the time of ovarian senescence, but cells will present differently from in which ovaries are removed. Aged mice will exhibit persistent vaginal cornification (PVC), in which densely packed, cornified epithelial cells and leukocytes will be present (Figure 2F). This can sometimes be confused for metestrus, but the key aspect that differentiates PVC is the lack of cycling. Thus, swabs must be taken on multiple consecutive days.

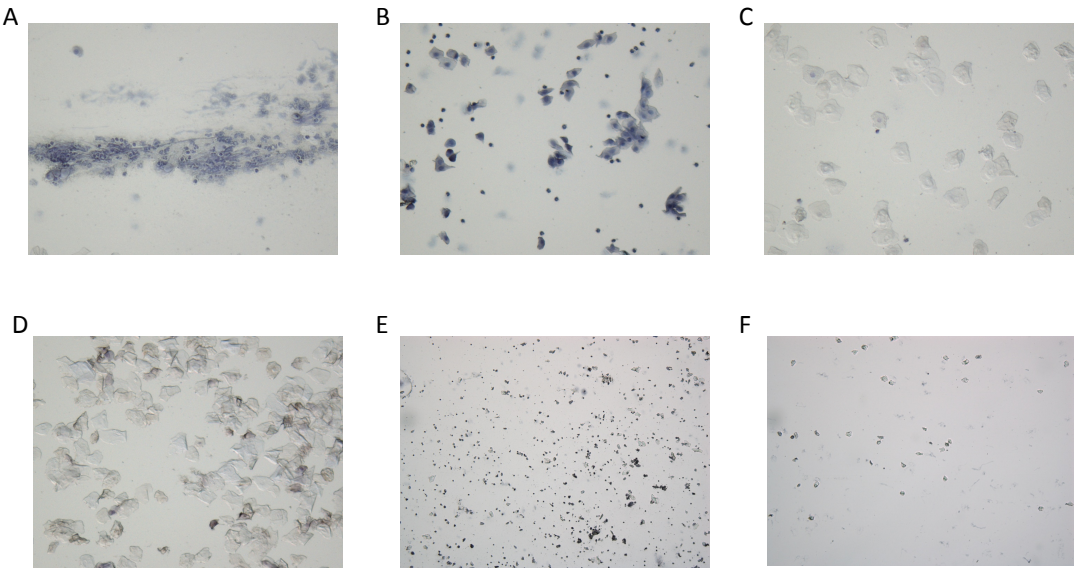

Figure 2: vaginal cytology slides from mouse (A-D) 10x magnification of the four phases of the estrous cycle; (E) 5x magnification slide from an ovariectomized mouse; (F) 5x magnification from an ovarian senescent aged mouse.

Ovariectomy

Ovariectomy was a critical tool for inducing a state of chronic estradiol deficiency in mice and was used in the majority of the studies in this dissertation. This procedure allows for estradiol deprivation to be studied, independent of aging. Furthermore, when combined with replacement of estradiol via an implanted hormone pellet, it allows for the targeting of estradiol by removing all other ovarian hormones (e.g., progesterone which is the other major female sex hormone produced by ovaries).

Bilateral ovariectomy was performed as follows on anesthetized mice for the purpose of removing the main estradiol-producing tissue and invoking a state of estradiol deficiency. Mice were anesthetized under isoflurane 1-2% mixed with oxygen at 125-250 mL/min while lying on a heating pad at 37° to maintain body temperature for the duration of the surgical procedure. Due to their small body size, mice are unable to independently maintain body temperature while under anesthesia. As such, a heating pad must be

employed to prevent mice from becoming hypothermic. Mice were given ophthalmic ointment to maintain moisture to the eyes during anesthesia and a sub-cutaneous injection of slow-release analgesic buphrenorphine (0.1 mg/kg) to ease any discomfort or pain associated with the surgical procedure and healing of the incision site during the days following surgery. The surgical site was prepared by shaving the hair on the back just below the iliac crest down to the lower ribs followed by cleansing with betadine and ethanol to create an aseptic site for the skin incision. An incision, approximately 2 cm, was made down the spine, and the skin fascia teased back to expose the underlying muscle. The ovaries were accessed through two incisions made in the muscle layer of the abdomen. The ovaries were located and the uterine tubes cauterized to remove the ovarian tissue. Care was taken to avoid directly gripping the ovary with the forceps as this could release a small amount of ovarian tissue back into the abdominal cavity and impact effectiveness of the surgery as residual ovarian tissue can still produce hormone. The muscle layer was closed with 6-0 silk suture and the skin closed with steel wound clips [107]. The wound clips were later removed, once the skin had healed, approximately 7-10 days post surgery.

Estradiol treatment

In order to isolate the effects of estradiol from other ovarian hormones, following the ovariectomy procedure, an implanted slow-release 17-β estradiol pellet (Innovative Research of America, Sarasota, FL) was utilized. 17-β estradiol was the specific estrogen used for treatment, as it is the most bioavailable form of estradiol. The dose of estradiol chosen varied from one study to another, depending on the question to be addressed and tolerability of the hormone dose by the mouse. Previous studies have utilized 0.18mg, 60 day slow release pellets in adult mice [14, 15, 217] which delivers approximately 3µg of estradiol per day, and 0.09mg, 60 day slow release pellets in aged mice which delivers approximately 1.5µg of estradiol per day. A lower dose is used for aged mice as studies report urinary retention when higher levels are used [17, 218].

For studies performed in adult female mice (4-6 months of age), a dose of 0.18mg released over the course of 60 days was used. For studies investigating longer duration of hormone deprivation, a lower dose pellet of 0.135 mg released over 90 days was used. This lower does was chosen to help mitigate bladder problems that can arise from the longer treatment duration and to correspond with the dose given to aged mice in previous studies [17, 219-222]. Thus, adult and aged female mice with 3 months of hormone deprivation were implanted with the lower dose 90-day release pellet. Important to note is that the relative higher and lower doses of estradiol treatment used in the studies in this dissertation both result in physiological level of circulating hormone.

In subsets of mice that underwent the ovariectomy procedure, estradiol was replaced through the use of a commercially available, slow-release estradiol pellet. While under anesthesia immediately following ovariectomy, the base of the neck was swabbed with betadine and the estradiol pellet implanted subcutaneously using a trochar at the base of the neck. This location was selected to help prevent the mouse scratching at the pellet and incision site during its normal grooming behaviors. The small incision site made by the trochar was sutured shut with 6-0 silk suture to ensure that the pellet did not become dislodged while the skin healed.

In vivo skeletal muscle contractile testing

A notable strength of the muscle physiology testing performed in this dissertation is the use of an *in vivo* approach (Figure 3). While studies investigating muscle strength *ex vivo* are more commonly found, and have the positive aspect of isolating the muscle from the environment, this can also be considered a weakness of testing only *ex vivo.* By utilizing an *in vivo* approach, the muscle is tested under native conditions and contributions from changes that may arise in vasculature, muscle innervation, neuromuscular junction, and membrane potential are observed in the muscle strength outcomes.

A

B

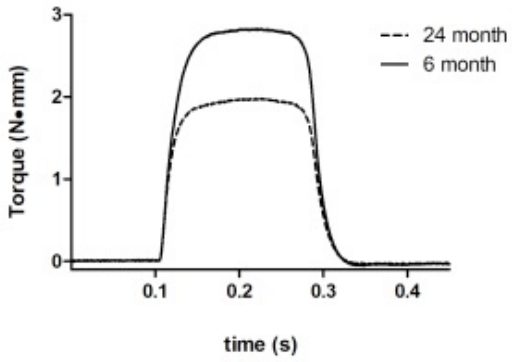

Figure 3: A) in vivo electrode positioning for recruitment of the posterior hindlimb muscles; B) Example tracing for posterior maximal isometric torque of an adult and aged female mouse.

For all *in vivo* testing protocols, mice were anesthetized with isofluorane (1-2% at oxygen flow rate of 100-200 mL/min), hair was removed from the anterior lower hind limb with Nair and the injury area aseptically prepared with betadine and ethanol. Ophthalmic ointment was applied to maintain moisture to the eyes while under anesthesia and mice were maintained on a heating pad at 37°C [107, 223]. Body temperature has a drastic impact on muscle performance, as muscles are critical to providing body heat through thermogenesis. Thus, a single degree drop in body temperature can drastically alter strength.

For testing of the posterior crural muscles (gastrocnemious, plantaris, soleus muscles), it is not possible to isolate the tibial nerve, thus denervation of the common peroneal nerve

was performed to eliminate co-activation of the antagonist muscles (anterior crural muscles) and allow for stimulation of the sciatic nerve [224, 225]. Denervation was accomplished as follows: the left leg of the anesthetized mouse was stabilized and an incision, approximately 0.25 cm in length, was made over the lateral crural ligament of the knee joint. The underlying hamstring muscle was resected, and the common peroneal and tibial nerves exposed, just distal to the branching of these from the sciatic nerve. The common peroneal nerve was severed and a portion of the nerve removed to eliminate antagonistic contractions of the anterior crural msucles . The skin was then sutured shut with 6-0 silk suture to keep underlying tissues moist during muscle testing.

Mice were positioned on their right side, with the left leg stabilized at the knee by a clamp and the left foot secured to a custom made foot plate attached to a servomotor that is attached to a force transducer (300B-LR; Aurora Scientific, Ontario, Canada). The foot and knee were secured so as to create a 90° angle at the knee and ankle joints thus aligning the tibial bone perpendicular to the foot plate. The servomotor can be moved through 40° range of motion about the ankle, 20° plantarflexion and 20° dorsiflexion. Muscle torque, force produced about the angle of the ankle, was measured by the force transducer. Platinum subdermal needle electrodes were placed to stimulate the sciatic nerve, for posterior testing. The needle electrodes were attached to a stimulator and stimulus isolation unit (E2-12, S28, and SIU5; Grass Telefactor, Warwick, RI). Stimulation parameters varied depending on the protocol, and are outline in the individual protocols below:

1. Peak isometric torque

   The peak isometric torque protocol is used not only to measure maximal strength but also to identify optimal voltage for all subsequent *in vivo* protocols. Voltage was increased from 3 to 8 V, and optimal voltage was defined as the voltage at which torque ceased to increase. The stimulator was set to parameters of 150-ms contraction duration of 0.5-ms square-wave pulses delivered at a frequency of 250 Hz.

2. Torque-frequency protocol

   The torque-frequency protocol is an important tool for identifying the effect of interventions (for the purpose of this dissertation those interventions are estradiol deficiency and age) on muscle excitability. While all other *in vivo* protocols stimulate at an optimal frequency to create maximal contractions, identified by previous studies, this protocol measures the torque produced across a range of stimulation frequencies. At low frequencies, multiple twitches of the muscle are observed and submaximal forces measured, as stimulation is not sufficient to cause fusion of twitches into tetanus. However, as frequency increases, twitches become more rapid and fuse to form the tetanic contractions and higher forces until complete fusion occurs – this is the optimal frequency.

   Data can be plotted as a percent of the maximal (or optimal) torque produced and shifts in the curve can be assessed. A leftward shift in the force-frequency curve indicates that lower frequencies are required to elicit the same percentage of maximal torque, or in other words the muscle is more excitable. A rightward shift indicates the opposite, that more stimulation is required to generate the same percent of maximal torque, a less excitable muscle. Another measure of muscle excitability is referred to as "Freq50", this is the frequency required to elicit 50% of maximal torque. The sciatic nerve was stimulated with optimal voltage and torque was measured at frequencies of 10, 20, 30, 40, 60, 80, 100, 125, 150, 200, 250, and 300 was measured.

3. Torque-velocity (Power) protocol

   The torque-velocity protocol was a key tool to address the objectives of this dissertation. Power, the product of torque produced at a given velocity, is widely understudied, especially in rodent models. Yet, it is known to be a better predictor of function and mortality than other muscle measurements [226].

During this protocol, the sciatic nerve was stimulated at 250 Hz while the leg was moved through the full range of motion at velocities of 100, 200, 400, 600, 800, 1000, and 1200 m/s. Power was calculated from the torque output of the muscle group at each velocity.

Results –

Uterine mass confirmed successful ovariectomy and estradiol treatment, although low-dose estradiol treatment did not return uterine masses to levels observed in control mice. Ovariectomy reduced uterine masses from 116.7 mg to 12.8 mg in adult mice and from 114.3 mg to 26.3 mg in aged mice. Treatment with estradiol increased uterine masses to 36.1 mg in adult mice and 56.4 mg in aged mice.

There was no main effect of age on peak isometric torque of the posterior muscles ($p=0.161$), with both adult and aged mice producing approximately 14.5 N□mm of torque. Ovarian hormone status also did not exhibit a main effect on peak isometric torque production ($p=0.165$; Figure 4).

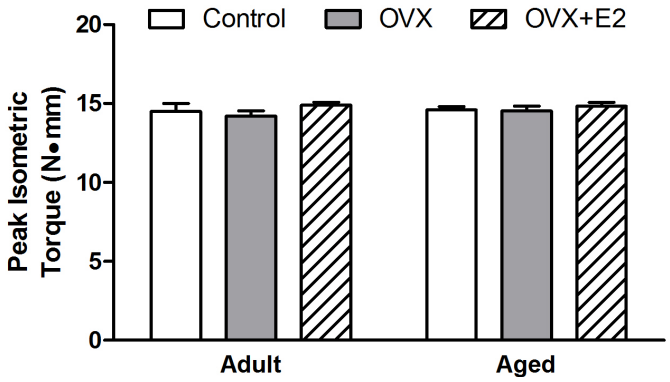

Figure 4: Age and ovarian hormone deprivation do not impact the peak isometric torque produced by the posterior crural muscles in female mice. Values are represented as mean ± SEM.

To further probe the impact of aging and estradiol on muscle, sub-maximal torque was measured at stimulation frequencies ranging from 10-300 Hz. There was a main effect of

age on submaximal torque at stimulation frequencies of 60 Hz and lower ($p \leq 0.039$) but not at stimulation frequencies of 80 Hz and higher ($p \geq 0.116$). At frequencies of 10-60 Hz, aged mice produced up to 20 % more torque than adult mice (post hoc $p \leq 0.039$). There was no main effect of ovarian hormone status on sub-maximal torque production at any stimulation frequency 10-300 Hz ($p \geq 0.085$; Figure 5).

Another method of looking at sub-maximal torque, freq50, compares the stimulation frequency necessary to elicit 50% of the maximal torque. Consistent with the analyses of torque across the stimulation frequencies, there was a main effect of age on freq50 ($p=0.013$). A stimulation frequency of 40.712 Hz was required in the aged mice while 44.855 Hz was required in adult mice to generate 50% of maximal torque. There was no difference in freq50 among the hormone groups ($p=0.697$).

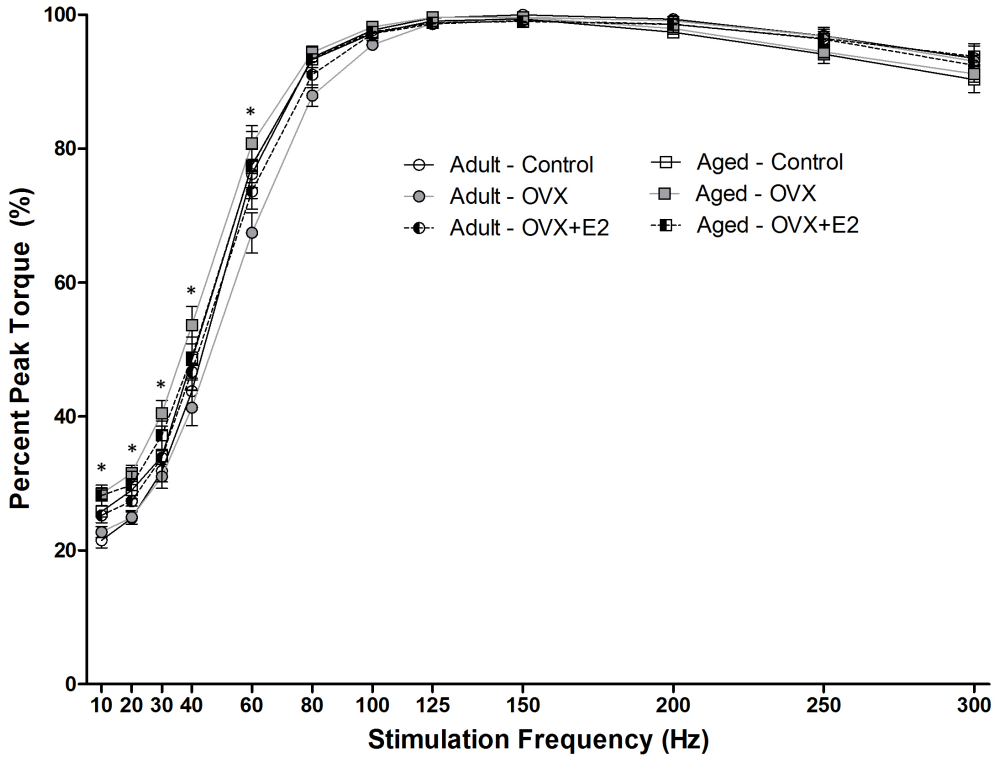

Figure 5: Age but not ovarian hormone deprivation impacts sub-maximal torque production of posterior crural muscles in female mice at frequencies of stimulation below 80Hz. Values are represented as mean ± SEM. * indicates significant effect of age.

There was no main effect of age ($p \geq 0.174$) or ovarian hormone status ($p \geq 0.542$) on power production as calculated from torque produced at velocities ranging from 100-1200 degrees/second (Figure 6).

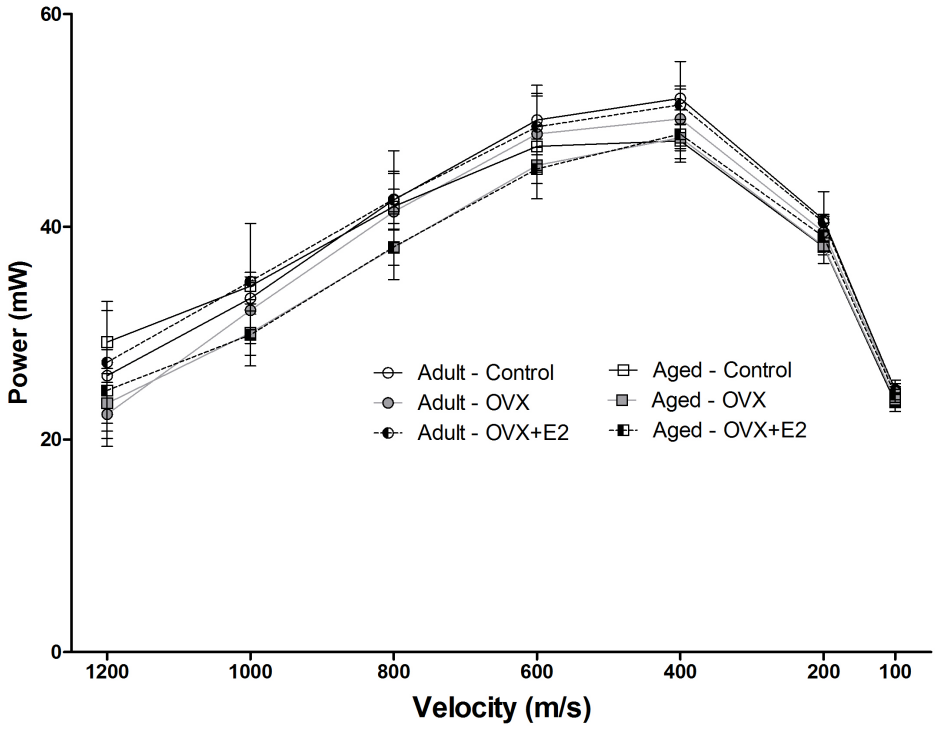

Figure 6: Age and ovarian hormone deprivation do not impact power production of posterior crural muscles in female mice. Values are represented as mean ± SEM.

Conclusions –

Removal of ovarian hormones by ovariectomy does not affect maximal strength, submaximal strength, or power of the posterior crural muscles in 6 or 24 month-old female mice. Furthermore, treatment of ovariectomized mice with 17-β estradiol for 10-12 weeks does not impact posterior crural muscle strength or power in adult or aged female mice. Female mice aged 24 months do not demonstrate decrements in maximal strength or power compared to 6 month-old adult mice; however, at low stimulation

frequencies, posterior crural muscles of aged mice generate more torque than adult mice. This difference may reflect a shift in motor unit types.

In general, results of this study do not support the hypotheses that muscle strength and power would decline with aging. As previously stated, female mice undergo ovarian senescence around 18-20 months of age. Thus the 24-month aged females used in this study had not been senescent for more than 6 months. Perhaps longer duration of ovarian hormone deficiency is necessary to elucidate the decrements in muscle function that have been previously demonstrated *ex vivo.* As longer duration of ovariectomy is not advisable in aged mice, an additional study was undertaken using adult female mice with a longer duration of ovarian hormone deficiency.

Study #1 - Part B:

Hypothesis –
Muscle power and submaximal strength will decrease with an extended duration of ovarian hormone deprivation.

Study Design –
Female C57BL6 mice aged 3-4 months were obtained from the National Institute of Health Aging Colony and Jackson Laboratories. Mice were ovariectomized and maintained for 6 months (n=5) or 12 months (n=5) in a hormone deficient state. Two of the mice in the 12-month ovariectomy group had to be euthanized prior to the endpoint due to health concerns. For comparison, ovariectomized mice from part A (n=10) were included in the analysis (3 month OVX). This was done to minimize the number of mice needed for the additional investigation. Vaginal cytology was performed for 4 consecutive days following recovery from surgery to verify effectiveness of ovariectomy.

As described in detail in the methods section of Study #1- part A, mice were anesthetized and *in vivo* muscle function was assayed. Maximal isometric torque, submaximal isometric torque (via torque frequency), and power (via torque velocity) of the posterior crural muscles were measured. Following *in vivo* testing, muscles were harvested and stored at -80°C for further biochemical analysis to address oxidative stress questions in a subsequent study. Uteri were weighed as secondary confirmation of effectiveness of ovariectomy.

Statistics –
Statistical power calculations were used to determine ideal sample sizes for studies necessary to apply an alpha level of 0.05 with 80% power. Expected variability was determined from previous studies performed in our lab and the wider literature base. Data were analyzed using a one-way ANOVA and a Holm-Sidak post-hoc test was

implemented to determine significant differences between groups. All statistical calculations were performed on Sigma Stat statistical software.

Results –

There was no difference between 3 months, 6 months, and 12 months of ovarian hormone deficiency on uterine mass, maximal isometric torque, sub-maximal isometric torque, or power (Table 1).

Parameter	3 month OVX (n=10)	6 month OVX (n=5)	12 month OVX (n=3)	one-way ANOVA p-value
Uterine mass (mg)	12.8 (3.3)	23.0 (8.9)	24.3 (10.1)	0.011
Peak isometric troque (N•mm)	14.2 (1.1)	15.2 (0.4)	13.9 (0.7)	0.140
Isometric torque (N•mm) Frequency: 10 Hz	3.1 (0.5)	3.4 (0.4)	3.0 (0.5)	0.417
20 Hz	3.4 (0.6)	3.8 (0.4)	3.6 (0.5)	0.335
30 Hz	4.2 (0.9)	4.8 (0.4)	4.8 (0.7)	0.316
40 Hz	5.6 (1.4)	6.4 (0.9)	6.6 (1.0)	0.372
60 Hz	9.2 (1.6)	10.3 (1.1)	10.8 (1.2)	0.183
80 Hz	12.0 (1.3)	13.5 (0.9)	12.6 (0.7)	0.062
100 Hz	13.0 (1.2)	14.2 (0.9)	13.3 (0.6)	0.165
125 Hz	13.4 (1.2)	14.5 (0.8)	13.5 (1.0)	0.224
150 Hz	13.6 (1.3)	14.6 (0.8)	13.7 (0.8)	0.239
200 Hz	13.5 (1.3)	14.5 (0.9)	13.4 (1.0)	0.292
250 Hz	13.2 (1.3)	14.0 (0.9)	13.0 (1.0)	0.393
300 Hz	12.7 (1.2)	13.3 (0.8)	12.4 (1.0)	0.488
Power (mW) Velocity: 1200 m/s	22.4 (9.5)	32.6 (14.5)	35.8 (22.3)	0.217
1000 m/s	32.1 (9.9)	38.7 (14.9)	36.8 (15.4)	0.600
800 m/s	41.4 (11.3)	47.8 (12.2)	40.9 (3.5)	0.534
600 m/s	48.7 (11.3)	54.6 (8.6)	45.9 (4.6)	0.439
400 m/s	50.1 (8.8)	54.4 (5.5)	48.0 (5.3)	0.472
200 m/s	39.5 (4.7)	40.9 (2.8)	38.6 (3.5)	0.718
100 m/s	24.5 (2.4)	22.9 (3.6)	24.2 (2.2)	0.584

Table 1: Posterior muscle performance measures and uterine mass from female mice ovariectomized for 3, 6, or 12 months. Values are represented as Mean (SEM).

Conclusions –

The duration of ovarian hormone deficiency does not appear to impact muscle function in adult female mice. However, it was not possible to include aged mice in this study, due to the difficulty of obtaining aged mice and successfully maintaining them for 6-12 months after ovariectomy. Therefore, it cannot be assumed that these results translate to aged female mice. Research in C57BL6/J male mice has shown that age-related strength loss and power deficits are not drastic until 28 months of age [56, 81-83]. Because the aged mice used in the first part of this study were only 24 months old, it is possible that they were not old enough to see drastic impacts of aging on skeletal muscle. Previous work by our laboratory in female rodents has focused on mice that are 28 months of age [17], as such it was unknown if at 24 months these mice would exhibit age-related changes in skeletal muscle. Additionally, by 28 months of age mice will have been ovarian senescent for a minimum of 6-8 months, thus further clarifying the impact of a longer duration of hormone deficiency in aged mice. It appears that at 24 months of age, female mice do not exhibit the strength or power loss associated with aging. To further probe the impact of aging on muscle function, an additional study was undertaken using 28-month-old female mice.

Study #1 - Part C:

Hypothesis –

Aging beyond 24 months will result in lower muscle power and submaximal strength.

Study Design –

Female C57BL6 mice aged 28 months (n=8) were obtained from the National Institute of Health Aging Colony and Jackson Laboratories. For comparison, control mice aged 6 months (n=6) and 24 months (n=8) from part A were included in the analysis. This was done to minimize the number of mice needed for the additional investigation and because of the limited availability of and cost to house aged mice. Vaginal cytology was performed for 4 consecutive days to verify normal estrous cycling in 6-month-old mice and ovarian senescence in 24 and 28-month-old mice.

As described in detail in the methods section of Study #1- part A, mice were anesthetized and *in vivo* muscle function was assayed. Maximal isometric torque, submaximal isometric torque (via torque frequency), and power (via torque velocity) of the posterior crural muscles were measured. Following *in vivo* testing, muscles were harvested and stored at -80°C for further biochemical analysis to address oxidative stress questions in a subsequent study.

Statistics –

Statistical power calculations were used to determine ideal sample sizes for studies necessary to apply an alpha level of 0.05 with 80% power. Expected variability was determined from previous studies performed in our lab and the wider literature base. Data were analyzed using a one-way ANOVA and a Holm-Sidak post-hoc test was implemented to determine significant differences between groups. All statistical calculations were performed on Sigma Stat statistical software.

Results –

There was no main effect of aging on maximal isometric torque of the posterior crural muscles (p=0.131, Figure 7).

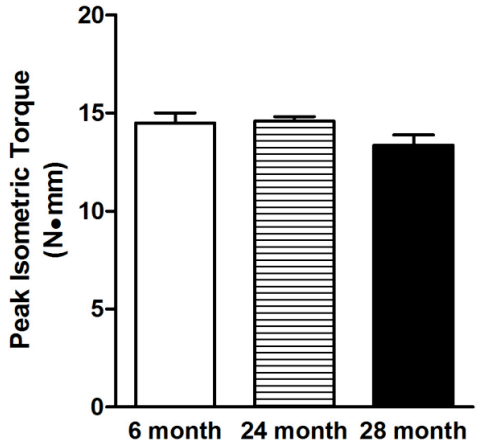

Figure 7: Age does not impact peak torque produced by posterior crural muscles in female mice. Values are represented as mean ± SEM.

There was no main effect of aging on sub-maximal torque ($p \geq 0.050$, Figure 8).

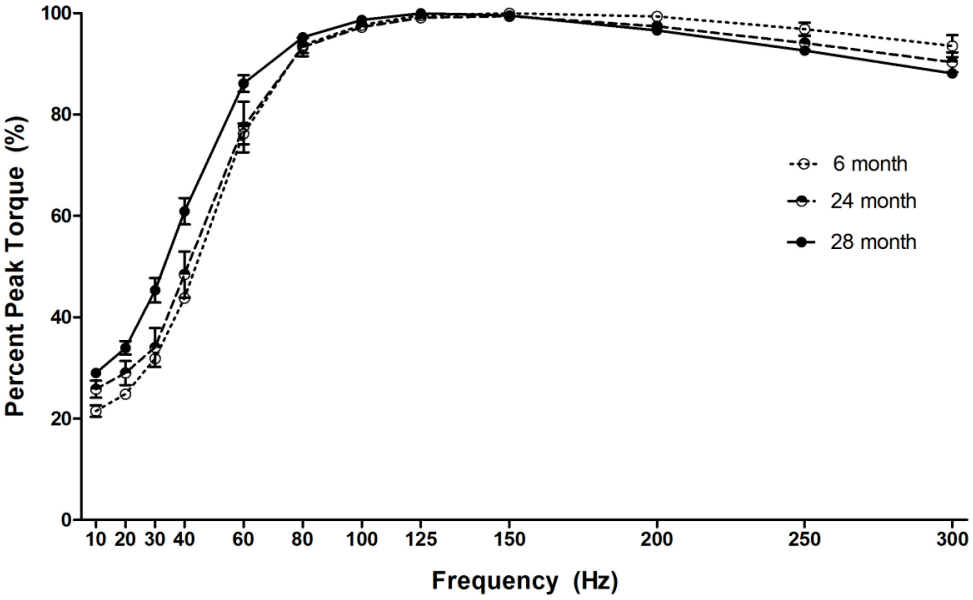

Figure 8: Age does not impact sub-maximal torque produced by posterior crural muscles in female mice. Values are represented as mean ± SEM.

There was a main effect of aging on torque produced while the muscle was being shortened at velocities of 100 - 1000 m/s ($p \leq 0.043$). Female mice aged to 28 produced 23 – 46 % less torque than 6 month or 24 month-old mice (post-hoc $p \leq 0.029$, Figure 9).

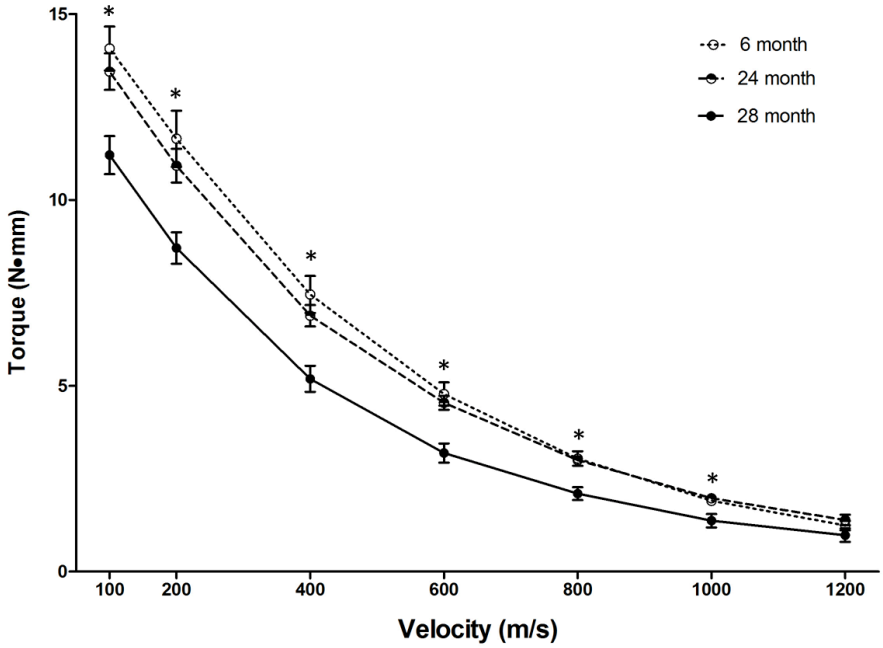

Figure 9: Posterior crural muscles of 28 month-old female mice produce less torque at velocities ≤ 1000 m/s compared to 6 month and 24 month-old female mice. Values are represented as mean ± SEM. * indicates significantly different from 6 month and 24 month old mice.

There was a main effect of aging on power production at velocities of 1000 m/s and slower ($p \leq 0.043$). Female mice aged to 28 months have lower power than 6 month or 24 month-old mice (post-hoc $p \leq 0.029$, Figure 10).

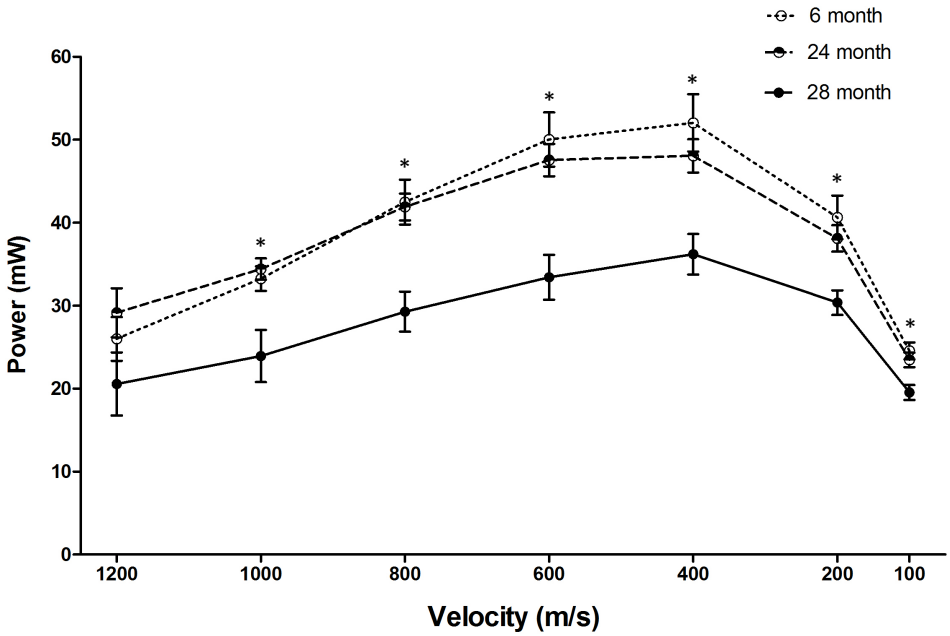

Figure 10: Posterior crural muscles of 28 month-old female mice produce less power at velocities ≤ 1000 m/s compared to 6 month and 24 month-old female mice. Values are represented as mean ± SEM. * indicates significantly different from 6 month and 24 month old mice.

Conclusions –

Aging to 28 months does not impact maximal or submaximal strength of the posterior crural muscles in female mice. This is in contrast to the results of part A, where 24-month-old mice exhibited higher submaximal strength at stimulation frequencies below 80Hz. However, in this study 28 month old mice produced less torque during contractions performed at velocities up to 1000 m/s. This resulted in lower power production of posterior crural muscles of 28-month-old females compared to 6 and 24-month-old females. These results are suggestive of an intrinsic difference in the contractile apparatus of muscles from 28-month-old mice that is not observed in 24-month-old mice. One possible explanation is that by 28 months of age, mice have been in a state of ovarian senescence for at least 6 months. Perhaps the differences observed between mice at 24 and 28 months of age result from the longer duration of hormone

deficiency in the older mice. Although longer duration of ovariectomy did not result in strength or power deficits in adult mice, perhaps low levels of ovarian hormones is of greater consequence to muscle function when combined with aging. A second possible explanation is that key aging-related changes in muscle may occur between 24 and 28 months of age, although this remains to be verified. Collectively, the results gleaned from 28-month-old mice provide valuable insight into changes in the ability of skeletal muscle to perform at velocity that occurs with aging in females.

Chapter Discussion –

Study #1 sought to address the impact of ovariectomy on the more physiologically relevant measures of sub-maximal muscle strength and muscle power, determine to what extent estradiol is involved in ovariectomy-induced alterations in muscle function, and to identify to what extent aging effects occur independent of hormone-driven alterations. The results of study #1 indicate an estradiol-independent effect of aging on skeletal muscle power. These results are in agreement with the literature in male rodents that demonstrate a loss in skeletal muscle function by 28 months of age [81, 82, 227, 228]. Furthermore, the results of the present study suggest that deprivation of ovarian hormones may not be the driving cause for loss of muscle power. Previous studies utilizing aging female rodents investigated muscle strength *ex vivo* [4, 17, 229]. There are several differences between this study and previous studies done by our laboratory, perhaps the most important of which is the choice of the posterior crural muscles. The majority of the previous investigations into the effects of estradiol on muscle function utilized the anterior crural muscles. The posterior muscles were chosen for investigation due to the larger contribution of these muscles the rodent ambulation as well as the more mixed fiber composition of this compartment compared to the anterior.

The results of study #1 did not support as drastic of an age-related strength loss as would be suggested by the literature in male rodents [81, 82, 227, 228] and further supports that males and females have different responses to aging. As previously discussed in the literature review, results from human literature would suggest that females would be more impacted than males, such that females would have lower strength with aging compared to age-matched male counterparts. One of the reasons behind this hypothesis is the loss of ovarian hormones that are thought to occur following ovarian senescence. However, recent research has shown that in contrast to our beliefs, estradiol levels may actually elevate at the time of ovarian senescence, rather than decline. If this is in fact the case, that the 24 and 28-month-old mice have high levels of estradiol, then the impact of hormones cannot be completely ruled out.

Overall, the results of study #1 demonstrate an impact of age on muscle function. Previous studies have supported a role of estradiol in muscle function as well, even though that is not corroborated by the results of this study. The underlying causes of the decline in function are not well understood. The strongest support comes from research suggestive of alterations in the binding of myosin. Evidence for oxidative changes in the contractile proteins of muscle, while not yet thoroughly investigated, is promising. The impact of aging on oxidative changes in contractile and non-contractile proteins of skeletal muscle is minimal, and merits further consideration. Initial studies in male rodents have identified changes in myosin structure [60, 62, 230] that impact its ability to produce force. However, studies investigating this in the context of females and with the impact of ovarian hormones have yet to be conducted. In order to elucidate the impact of aging and estradiol on oxidative stress and damage globally as well as at the level of the skeletal muscle proteins, additional studies were undertaken.

Chapter #3: Mechanism of aging and estradiol-related changes in skeletal muscle contractile function – alterations in oxidative damage

Studies investigating the effects of aging on skeletal muscle oxidation in females are few. Aging has been shown to result in oxidative alterations to contractile proteins, such as myosin [62, 65, 230-232], that impacts the muscle's ability to form cross-bridges necessary for muscle contraction. However, as with most aging research, the bulk of this work has been completed in males. And as demonstrated in other work investigating age-related alterations in skeletal muscle in both males and females, there are sex differences in some aspects of aging [63, 233-241]. Thus, it is possible that sex-differences in oxidation of skeletal muscle occur and due to the disparate effects of aging on muscle strength in males versus females, investigation of sex differences is vital. The following studies seek to determine not only the sex-differences present in skeletal muscle oxidation but also the impact of ovarian hormone deprivation in adult and aged female mice. Other research in rodents indicates alterations in oxidative stress under aging and disease conditions in males. Questions that will be answered in the next serious of studies are: Are there changes in global oxidative stress levels induced by ovarian hormone deprivation and aging in females? Is there oxidative damage sustained by the contractile proteins following exercise in aged, ovarian senescent female mice?

Study #2 – Part A:

Hypothesis –

Levels of global oxidative stress in female mouse muscle will increase with age.

Study Design –

Urine samples were non-invasively collected from female C57BL6 mice aged 4 months (n = 10), 14 months (n =8), and 28 months (n = 8). Mice were obtained from the National Institute of Health Aging Colony and Jackson Laboratories, and were utilized for other studies reported in this dissertation. Levels of urinary biopterin, a marker of global oxidative stress, were measured and normalized to urinary creatinine levels as described in the methods below, with the exception that samples from 14 and 28-month-old mice were pooled with another mouse from their respective age groups due to smaller volume of urine collection.

Statistics –

Data were analyzed using a one-way ANOVA and a Holm-Sidak post-hoc test was implemented to determine significant differences among groups. All statistical calculations were performed on Sigma Stat statistical software.

Methods –

High Performance Liquid Chromatography – Biopterin Quantification

High Performance Liquid Chromatography (HPLC) was used to quantify biopterin (BP) and total biopterin ($BP+BH_2+BH_4$) in urine from mice. Biopterin (Neopterin in humans) is the non-enzymatic oxidation product of BH_2 that has been used as an indicator of oxidative stress in several disease states in both mice and humans [242-244]. Thus, urinary biopterin can be used to monitor perturbations in systemic oxidative status in response to various stimuli/stressors. Additionally, because BH_4 is an essential co-factor in the synthesis of nitric oxide (NO), serotonin, dopamine and epinephrine, urinary

quantification of total biopterin provides information regarding several necessary biosynthetic pathways. In this dissertation, urinary biopterin and total biopterin were measured as indicators of global oxidative stress and nitric oxide availability, respectively, with estradiol deprivation and aging.

To collect urine, mice were restrained and their bladders gently milked. Urine was collected in a microcentrifuge tube and stored at -20° until time of analysis. Sample preparation and analysis were completed according to the methods of Lindsay et al [245]. Samples were thawed on ice and diluted 1:50 in 20mM Ammonium Phosphate, pH 2.5. For total biopterin analysis, 100 µL of diluted sample was aliquoted into a separate microcentrifuge tube. Twenty microliters of acidic iodide (5.4 % I_2/10.8 % KI in 1 M HCl) was added to the sample to oxidatively convert BH_2 and BH_4 to biopterin. Following addition of the acidic iodine, samples were incubated for 15 minutes at room temperature in the dark, to avoid oxidative loss by UV. Following incubation, 10 µL of 0.6 M ascorbic acid was added to reduce the tri-iodine and 100 µL was transferred to an HPLC vial for analysis (Chromtech, MN, USA). Untreated diluted urine, biopterin standard (1 µM concentration) and creatinine standard (100 µM concentration) were also loaded into HPLC vials and analyzed by strong cation exchange (SCX) chromatography on a Shimadzu i-series 2030C with UV detector and autosampler, RF-20A florescence detector and on-line degasser with a 20 mM ammonium phosphate pH 2.5 mobile phase pumped isocratically at 1 mL/min. Samples were maintained at 4°C via the HPLC cooling unit and separated based on positive charge using a Luna 5 µm SCX 100 Å, 250 x 4.6 mm column (Phenomenex, CA, USA). Biopterin was detected at 353 nm excitation and 428 nm emission and creatinine at its natural absorbance of 234 nm. The concentration and identity of biopterin and creatinine were compared to their corresponding standard (freshly prepared each day and spaced evenly throughout each analytical run) and quantified by peak area using the software Shimadzu Class VP. Biopterin and total biopterin were normalized to creatinine and presented as mmol/mol to account for the individual variation in mouse hydration status that can significantly influence urinary metabolite concentrations [246].

Results –

There was no main effect of aging on urinary biopterin levels ($p = 0.056$). Female mice of all ages studied had biopterin levels between 0.6 and 1.2 mmol/mol creatine (Figure 11).

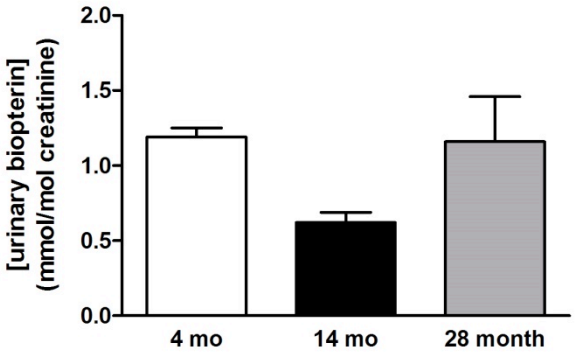

Figure 11: Aging does not impact levels of urinary biopterin.
Values are expressed as Mean ± SEM.

There was a main effect of aging on levels of total biopterin in the urine ($p<0.001$). Female mice aged 14 and 28 months had up to 67% higher levels of total biopterin than 4 month old mice (post-hoc $p \leq 0.002$, Figure 12).

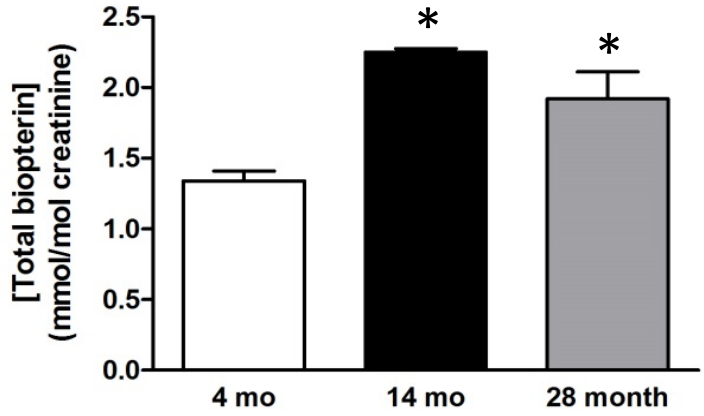

Figure 12: Aging results in increased levels of total biopterin in the urine of female mice. *indicates significantly different from 4 month old mice. Values are expressed as Mean ± SEM.

There was also a main effect of aging on the ratio of biopterin (BP) to total biopterin ($p < 0.001$). Female mice aged 14 months had 62% lower BP:total BP than 4 month old mice (post hoc $p \leq 0.022$); however, by 28 months of age the ratio has rebounded to only 29% lower than 4 month old mice (post hoc $p \leq 0.014$, Figure 13).

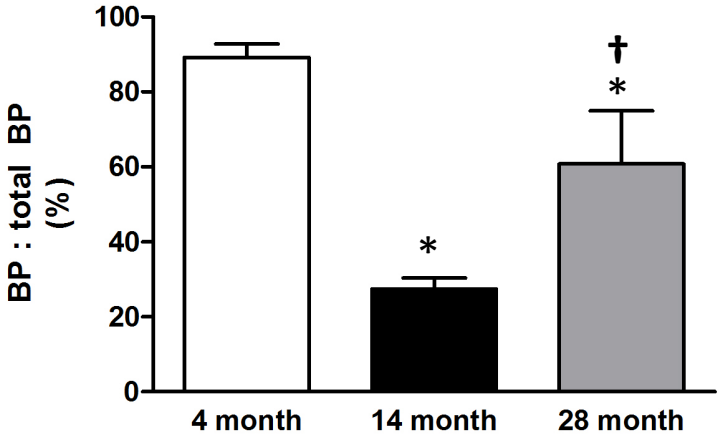

Figure 13: Aging results in a decreased ration of urinary biopterin to total biopterin (BP:total BP). * indicates significantly different from 4-month mice. † indicates significantly different from 14 month mice. Values are expressed Mean ± SEM.

Conclusions –

Aging resulted in increased total biopterin; however aging to 28 months did not alter levels of urinary biopterin, a measure of global oxidative stress. As a result, the ratio of urinary biopterin to total biopterin was low in mice aged 14 months compared to 4-month-old mice but that ratio partially rebounded by 28 months of age. The alteration in the ratio was driven by the increase in total biopterin and not a decrease in urinary biopterin. Levels of oxidative stress, as measured through oxidation of BH_2 to BP, appear to be less sensitive to aging than synthesis of BH_4. This indicates that with aging there is more de novo synthesis of BH_4, indicative of potential changes in NOS regulation. These results are further strengthened by results of previous studies showing increases in nitric oxide levels in skeletal muscle with aging [247]. The changes observed with aging in females beg the question of hormone-dependent effects, that is, might the differences be

due to ovarian senescence and the lack of estradiol production rather than, or in addition to, aging. To address the impact of ovarian hormone deficiency, an additional study was carried out with mice that had undergone ovariectomy.

Study #2 – Part B:

Hypothesis –

Levels of global oxidative stress in female mice will increase with ovarian hormone deficiency.

Study Design –

Urine was collected non-invasively from multiple groups of ovariectomized female mice used in study #1 – part B prior to *in vivo* testing and tissue harvest. Urine was collected from 3 groups of mice: 1 month (n = 5), 5 months (n = 7), or 10 months (n=4) following ovariectomy. Samples were assayed for urinary biopterin, a marker of oxidative stress, and normalized to urinary creatinine levels, as described in the methods of Study #2 – Part A. Urine from 6 month old control, ovary-intact females used in Study #2 – part A was used for comparison.

Statistics –

Data were analyzed using a one-way ANOVA and a Holm-Sidak post-hoc test was implemented to determine significant differences between groups. All statistical calculations were performed on Sigma Stat statistical software.

Results –

There was a main effect of ovarian hormone deficiency on levels of urinary biopterin ($p = 0.005$) in groups of mice that had been ovariectomized for 1, 5, or 10 months. Levels of biopterin were 62% lower in adult female mice following 5 months of ovariectomy compared to control (post hoc $p = 0.012$; Figure 14).

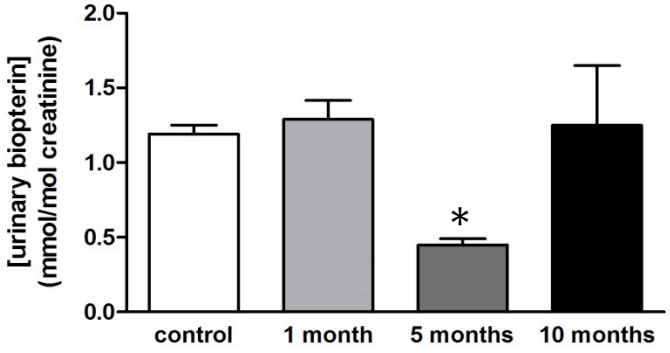

Figure 14: Ovariectomy resulted in lower levels of urinary biopterin at 5 months post surgery. * indicates significantly different from control. Values are expressed as Mean ± SEM.

There was a main effect of ovariectomy on levels of total biopterin ($p < 0.001$). Levels of total biopterin increased by more than 50% in all groups of ovariectomized mice (1, 5, and 10 months) compared to ovary-intact mice ($p < 0.001$; Figure 15).

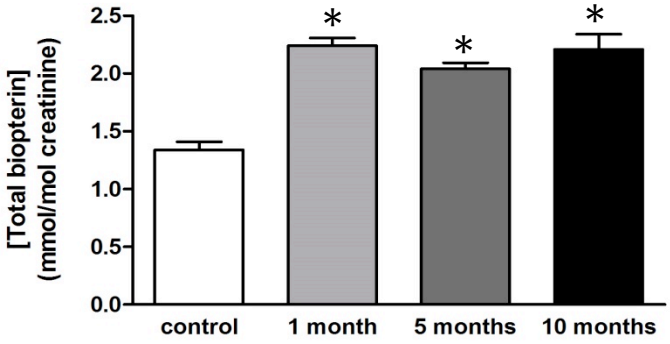

Figure 15: Ovariectomy resulted in higher levels of total biopterin compared to control female mice. * indicates significantly different from control. Values are expressed as Mean ± SEM.

There was a main effect of ovariectomy on the ratio of urinary biopterin to total biopterin ($p < 0.001$). In groups of mice that had been ovariectomized for 1, 5, or 10 months, the ratio was lower than that of control, ovary-intact mice ($p < 0.001$; Figure 16).

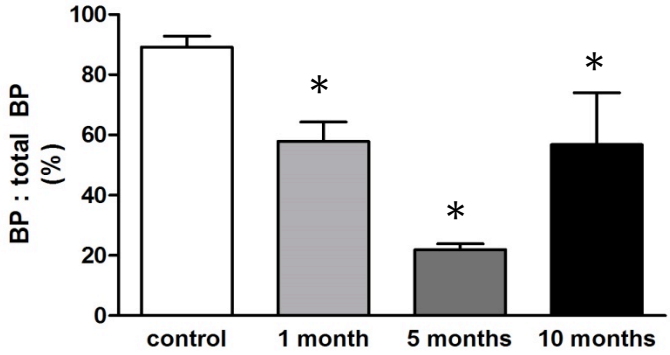

Figure 16: Ovariectomy resulted in a lower ratio of urinary biopterin to total biopterin compared to control female mice. * indicates significantly different from control. Values are expressed as Mean ± SEM.

Conclusions –

Levels of urinary biopterin were lower in mice that had been ovariectomized for 5 months. Levels of total biopterin were increased with ovariectomy, regardless of the duration of hormone deficiency. The magnitude of increase was similar to that observed with aging. Furthermore, similar to the aged mice, the ratio of urinary biopterin to total biopterin was lower in ovariectomized mice compared to control. Once again, the change in the ratio was driven more so by the increase in the BH_4 synthesis and less so by the alterations in urinary biopterin. However, it remains to be determined if the changes observed with aging are the result of hormonal changes or are driven independently by aging. Additional studies with aged mice of varying hormonal status would be necessary to identify hormone-independent effects of aging. Similar magnitude of increase in de novo synthesis is observed in ovariectomized mice as what was found in study #2 – part A for aged mice. Collectively, these results indicate that global levels of oxidation are not drastically altered by removal of ovarian hormones.

Previous research has indicated that tissue-specific oxidative damage occurs with aging and that oxidative modifications to contractile proteins of muscle may impact strength [62, 248-254]. Although changes in global oxidative stress are important to consider and can help inform what may be occurring in the muscle, direct measurement of oxidative changes to proteins in the skeletal muscle is necessary to further elucidate the mechanisms through which muscle function can be impacted. To further probe the influence of ovarian hormones on oxidative damage to skeletal muscle proteins, subsequent studies were carried out on male and female rodents. It is suggested that the response to exercise is a better measure of age-related changes than baseline alterations [149, 203, 255-257]. As such, studies were undertaken on male and female mice to identify the extent to which contractile and non-contractile proteins are oxidatively damaged following exercise. These studies address the following questions: Are there sex differences in oxidative damage to skeletal muscle with aging? Are contractile proteins or non-contractile proteins, such as cytosolic/mitochondrial/membrane proteins, more affected?

Study #3 – Part A:

Hypothesis –

Oxidative damage to skeletal muscle will be lower in adult females compared to males; however, with aging oxidative damage will increase greatly in females but not males such that aged females will have greater oxidative damage than aged males. Furthermore, levels of oxidative damage will be greater in the contractile proteins than in the cytosolic/mitochondrial/membrane proteins.

Study Design –

Male and Female C57BL6 mice aged 4 months (n = 4 of each sex) and 28 months (n = 5 of each sex) were obtained from the National Institute of Health Aging Colony. Mice were anesthetized and subjected to *in vivo* electrically stimulated muscle contractions of the left hindlimb muscles, as described in Study #1 – part A. Following the bout of *in vivo* exercise, mice were injected with Sodium Pentobarbital (100 mg/kg). Gastrocnemius muscles were excised approximately 30 minutes following the completion of the exercise bout. Muscles were flash frozen in liquid nitrogen and stored at -80°C prior to use for oxidative damage assays. The medial head of the frozen gastrocnemius muscle that had undergone the exercise protocol (left leg) was homogenized, fractionated, and assayed for oxidized proteins using the biotin hydrazide marker, as described in detail in the methods below.

Statistics –

Because expected variability was unknown at the time of the study, group sizes were determined based on what is commonly seen in the literature. Data were analyzed using a two-way ANOVA (age x sex). Since there were no statistically significant interactions, all p-values reported are main effects of age and sex. All statistical calculations were performed on Sigma Stat statistical software.

Methods –

Western Blots

Careful preparation of samples was necessary to avoid inducing oxidative modifications that can easily occur during the homogenization procedure. To avoid such oxidation, muscles were immediately frozen in liquid nitrogen after harvesting from the mouse and were stored at -80 ^{o}C until processed further. During all steps of the homogenization samples remained frozen as detailed below. Immediately after homogenization, muscles were transferred to a solution containing 20% trichloroacetic acid. The low pH prevents oxidation but is not so low as to induce other biochemical changes [258, 259].

Western blots were a vital tool for the studies of this dissertation addressing the cellular changes to muscle resulting from estradiol deprivation and aging. The use of western blots allowed quantification of muscle specific oxidative damage as well as quantification of antioxidant proteins of interest. Oxidative modifications are easily induced with traditional methods of sample preparation and as such diligent work went into developing techniques to minimize the impact of homogenization and sample preparation on the outcome measures of interest. Precise and careful sample preparation was developed and the final procedures for this sample preparation are as follows.

Muscle homogenization

At the time of sacrifice, gastrocnemious muscles were snap frozen in liquid nitrogen and stored at -80°C until time of assay, at which time muscles were homogenized in a frozen state as follows: a mortar and pestle were cooled with liquid nitrogen so that samples would remain frozen during the entire homogenization process. The frozen muscle was added to liquid nitrogen in the mortar and was ground into a frozen powder. Liquid nitrogen was added during the process and the muscle was never allowed to thaw. The frozen muscle powder was transferred to a microcentrifuge tube containing 1 mL of a homogenization buffer consisting of 1x phosphate buffered saline (PBS) with 1% sodium dodecyl sulfate (SDS; detergent) and 20% trichloroacetic acid (TCA). The acid

concentration of 20% has been shown sufficient to inhibit oxidation of proteins, but not so high as to induce other molecular changes to proteins. Muscle powder was vortexed in the buffer to ensure exposure to the acid, and then stored at -20°C.

Once all samples had been homogenized, it was necessary to remove the acid in order to perform the fractionation and enrichment of sample required for Western Blots, as the proteins will precipitate to some extent in the 20% acid solution. Acid was removed via a methanol precipitation; ice cold methanol was added to samples, which were then vortexed and frozen at -20°C for no less than 2 hours in order to precipitate the proteins. Samples were spun in a refrigerated 4°C centrifuge at >20,000g to pellet out all proteins. The supernatant was discarded and a pipette was used to remove any remaining liquid. Pellets were gently washed and re-spun to remove any remaining traces of acid. Pellets air-dried for 5 minutes prior to re-suspension in 1x PBS with 1% SDS buffer. To re-suspend pellets, it was necessary to sonicate the samples at 4°C using 1-2 second bursts for a total of 30-45 seconds per sample.

Following re-suspension, samples were spun at 1000*g* for 15 minutes to precipitate out the myofibrillar and nuclear fraction (here onward referred to as the myofibrillar fraction) [260]. The supernatant, which contains the cytosolic and membrane fraction (here onward referred to as the cytosolic fraction), was pipetted off and saved on ice in a new microcentrifuge tube. The myofibrillar fraction pellet was re-suspended in 1x PBS with 1% SDS via 30 seconds of vortexing, and saved on ice. Protein concentrations of all samples were measured on a nano-drop spectrometer. A 100 uL portion of each sample was diluted to a protein concentration of 2 mg/mL and 6x Laemmli sample buffer was added to help stabilize the proteins, and frozen at -20°C. Remaining sample volume was stored at -80°C. At the time of western blot analysis, fractions were assayed for proteins specific to each fraction (e.g., myosin and GAPDH) to ensure successful fractionation.

Oxidative damage blot and gel

Remaining undiluted homogenate samples were thawed on ice, a 200 uL aliquot was precipitated using methanol, as previously described, fractionated (to allow for separate analysis of oxidation in myofibrillar and cytosolic proteins) and re-suspended in a biotin hydrazide coupling buffer with phosphatase inhibitors, protease inhibitors, and SDS. Samples were treated with biotin hydrazde to a final concentration of 05.mM and incubated at room temperature for 2 hours while agitated, as adapted from Grimsrud et al [258]. A set of negative controls, not treated with biotin hydrazide, was also run. Following incubation, Lamelli sample buffer was added and samples were heated at 95C prior to loading on a 10% SDS-PAGE (electrophoresis) gel. 10uL of protein was loaded for each sample. Duplicate gels were run at 150V for 60 minutes at 4C and one set of gels subsequently transferred to PVDF membranes. The remaining set of gels was stained with coomassie for measurement of total protein.

Following overnight transfer to PVDF membranes at 30V and 4C, membranes were blocked with PBS-based Odyssey blocking buffer diluted 1:1 with PBS for 1 hour at room temp, rinsed with PBST, and incubated with IR800-conjugated Streptavidin (1:10,000) in PBST. Membranes were rinsed in 4x15min in PBST. Membranes and coomassie stained gels were then imaged on the LICOR 800 channel and analyzed for sample signal of the entire lane. Oxidized protein, as identified by IR800-streptavidin tag, was normalized to total protein, as identified by coomassie gel (Figure 17).

A

Biotin-Hydrazide tagged oxidized protein blot

B

Total protein: Coomassie stained SDS-Page gel

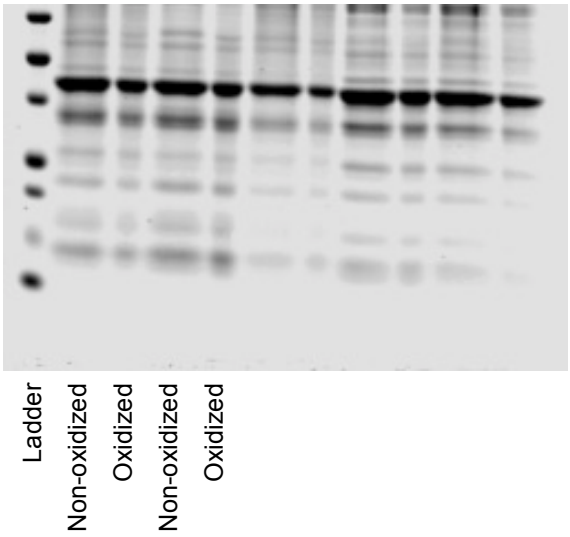

Figure 17: A) Biotin-Hydrazide tagged protein carbonylation blot and B) SDS-page total protein gel with positive (oxidized) and negative controls (non-oxidized)

Results –

There was a main effect of sex (p = 0.003); female mice had higher levels of oxidized cytosolic proteins in gastrocnemius muscle than male mice following exercise. There was no effect of age on levels of oxidized cytosolic proteins (p = 0.184, Figure 18).

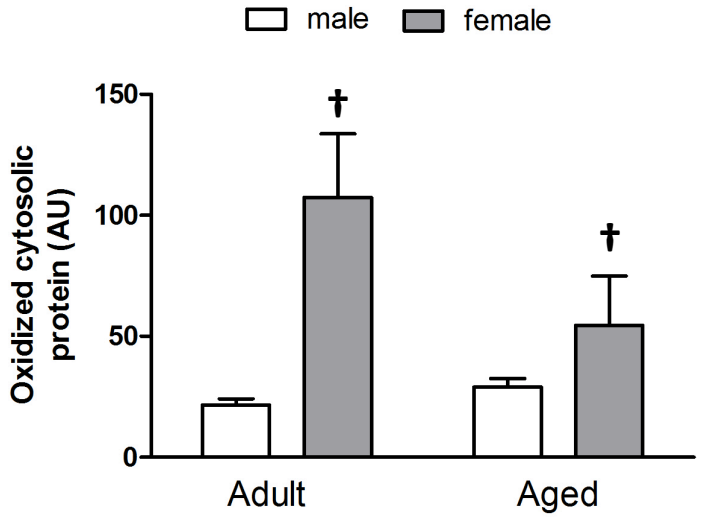

Figure 18: Oxidation of cytosolic proteins in the gastrocnemius muscle was higher in female than in males. Values are expressed as Mean ± SEM. † Indicates significantly different from male at same age.

There was no effect of sex the on the oxidation of myofibrillar proteins of the gastrocnemius muscle (p = 0.630). However, there was a main effect of age (p = 0.001). Aged mice had approximately 45% less oxidation than adult mice (Figure 19).

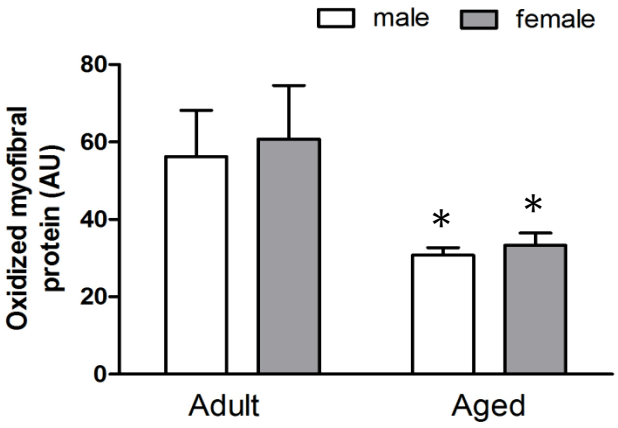

Figure 19: Oxidation of myofibrillar proteins in the gastrocnemius muscle declined with aging. Values are expressed as Mean ± SEM.
* Indicates significantly different from adult of same sex.

Conclusions –

Gastrocnemius muscles of female mice exhibited greater oxidation of cytosolic proteins (non-contractile) than males, regardless of age. Indicating a sex-dependent preferential oxidation of the proteins associated with cell viability. However, myofibrillar proteins (contractile) exhibited an age-dependent effect on oxidation that was not impacted by sex. These results led to the question, what are the contributions of ovarian hormones to the alterations in oxidative damage to muscles following contractions? Based on the results observed in this study, it is likely that ovarian hormones play a role. To further explore the direct effects of ovarian hormones on muscle protein oxidation, an additional study utilizing ovariectomized female mice was performed. This study investigated the age-independent effects of ovarian hormones on oxidation of proteins in skeletal muscle of adult and aged female mice.

Study 3 – Part B:

Hypothesis –

Removal of ovarian hormones will result in increased oxidative damage in skeletal muscles.

Study Design –

Adult female C57BL6 mice were obtained from the National Institute of Health Aging Colony at 3 months of age. Mice were randomly assigned to be maintained as controls (n=4) or to undergo ovariectomy. Mice that underwent ovariectomy were maintained for either 3 months (n=5) or 8 months (n=5) in a hormone deficient state. Vaginal cytology was taken for 4 consecutive days to verify successful ovariectomy. At the conclusion of the hormone deprivation, mice were anesthetized and subjected to *in vivo* electrically stimulated muscle contractions, as described in Study #1 – part A. Following the bout of *in vivo* exercise, mice were injected with Sodium Pentobarbital (100 mg/kg). Approximately 30 minutes post-exercise, gastrocnemius muscles were excised, flash frozen in liquid nitrogen, and stored at -80°C. At the time the oxidative damage assay was performed, the medial head of the frozen gastrocnemius muscle was homogenized, fractionated, and assayed for oxidized proteins using the biotin hydrazide marker, as described in detail in the methods section of Study #3- part A.

Statistics –

Because expected variability was unknown at the time of the study, group sizes were determined based on what is commonly seen in the literature. Data were analyzed using a one-way ANOVA and a Holm-Sidak post-hoc test was implemented to determine significant differences between groups. All statistical calculations were performed on Sigma Stat statistical software.

Results –

There was no effect of ovariectomy on the oxidation of cytosolic proteins (p = 0.729, Figure 20).

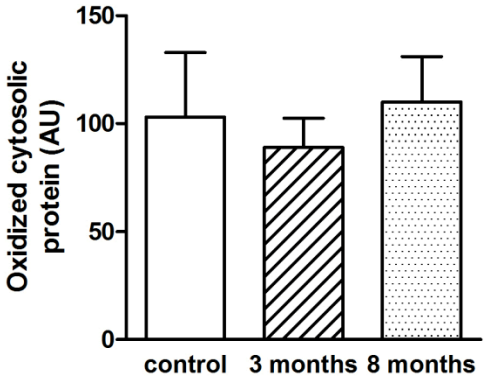

Figure 20: Ovarian hormone deficiency does not affect levels of oxidized cytosolic proteins in the gastrocnemius muscle of female mice. Values are expressed Mean ± SEM

There was an impact of ovariectomy on the oxidation of myofibrillar proteins (p = 0.028). After 8 months of ovariectomy, levels of oxidized myofibrillar proteins were 75% higher than control (post-hoc p=0.047, Figure 21).

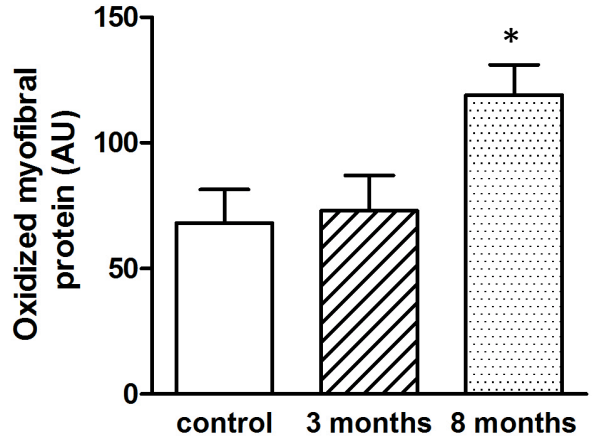

Figure 21: Eight months of ovarian hormone deficiency results in increased levels of oxidized myofibrillar proteins in the gastrocnemius muscle of female mice. Values are expressed as Mean ± SEM.* indicates significantly different from control.

Conclusions –

Ovariectomy did not impact oxidation of cytosolic proteins (non-contractile) and had the opposite effect on the oxidation of myofibrillar proteins (contractile) than anticipated based on the results of study #3- part A. Mice ovariectomized for eight months had higher levels of myofibrillar protein oxidation than control mice and mice ovariectomized for three months. This is in contrast to study #3- part A where male mice (no ovarian hormones) had lower levels of cytosolic oxidation than female mice and aged mice (ovarian senescent females) had lower levels of myofibrillar protein oxidation. The results of study 3 suggest that there are independent effects of age and ovarian hormone deficiency on protein oxidation in skeletal muscle.

Chapter Discussion –

Taken together, the results of studies #2 and #3 indicate that there are effects of both aging and ovarian hormones on oxidative stress and oxidative damage to muscle proteins. Specifically, under conditions of advanced age and ovarian hormone deficiency there is a robust increase in total biopterin, indicating greater de novo synthesis of BH_4, a necessary co-factor for NOS activity, and suggesting alterations in NOS handling when ovarian hormones are lacking. These results lend further support for a global impact of ovarian hormones on BH_4 and ultimately oxidative stress [261].

These studies also give evidence for sex differences in oxidation of cytosolic proteins that do not appear to be driven by female sex hormones. Previous studies focused on alterations with aging in males have yielded mixed results. Several have indicated minimal impact of aging on oxidative stress following exercise [149, 262, 263] whereas others have demonstrated age-associated changes [264-266]. The age-associated changes to skeletal muscle oxidation in females observed in study #3 are novel and contribute to our understanding of regulating factors of oxidation in skeletal muscle with aging. The protective effects of ovarian hormones on skeletal muscle observed in these dissertation studies are consistent with what would be expected based on the literature in other tissues, specifically that estrogens can be protective against oxidation [18, 172, 173, 267, 268]. Oxidation of proteins results from an REDOX system imbalance, either due to an increase in reactive oxygen and nitrogen species or from a decrease in antioxidants. There is minimal evidence to indicate which of these is the mechanism by which estrogens are protective; the latter possibility (i.e., antioxidants) was explored further in the studies reported in chapter 4.

Finally, the results of the studies in this chapter provide evidence for a decline in oxidation of myofibrillar proteins with aging, which is in contrast to much of what has been shown in proteins of other highly metabolic tissues such as the kidney and liver [269-271] and heart [272]. The reduced oxidation of myofibers with aging appears to be

independent of declining ovarian hormones as both males and female exhibit similar declines. Furthermore, protein oxidation was greater in muscles of ovariectomized females following eight months of hormone deprivation. Only a few studies have measured skeletal muscle protein oxidation in both female and male rodents; the results of which indicated increased oxidation with aging, particularly in male rats [273, 274]. Previous studies did not seek to investigate the differences in oxidation status between contractile and non-contractile muscle proteins, which could account in part for the discrepancies between results of those studies and the ones performed in this dissertation.

Higher levels of oxidation in contractile proteins, as shown in Figure 21, would suggest a resulting decrement in muscle strength and power; however, based on the results of study #1, such a force decrement was not observed. Several other studies seeking to investigate the interplay between oxidative damage and muscle function have also been unsuccessful in teasing apart the mechanisms through which sarcopenia and force loss occur [173, 253, 275, 276] . Nonetheless, the results of this study elucidate specifically the impact of ovarian hormone deficiency on oxidative damage to muscle proteins and provide a novel contribution to the field.

The disparate results of study # 3 following a long-duration of ovariectomy compared to short-term ovariectomy highlight the importance of investigating multiple time points and ages. However, without additional studies it is not possible to rectify the underlying causes of the altered REDOX mechanism that occurs following long-term hormone deprivation. Thus, to further investigate the impacts of aging and ovarian hormone deficiency at multiple ages on redox balance of skeletal muscle tissue, additional studies on key antioxidants were performed.

Chapter #4: Effects of aging and ovarian hormone deprivation on antioxidant protein expression in skeletal muscle

Previous research has provided evidence for an alteration of antioxidant adaptability in skeletal muscle with aging, but the impact of sex is not well established. Furthermore, minimal research has been undertaken to investigate the effects of estradiol and ovarian hormones on antioxidant response. Previous work in our laboratory [37] and others [277-281] has supported an impact of estradiol on gene regulation of antioxidants. However, the effects of age and hormonal status on antioxidant protein expression remain to be assessed and are arguably more critical to maintaining muscle redox balance than is gene expression. As such, the following studies investigate the impact of sex and estradiol on antioxidant protein expression in aged compared to adult mouse muscle. This information may help to explain results reported in the previous chapter of this dissertation, for example, why myofibrillar protein oxidation is elevated in muscle in response to 8 months of ovarian hormone deficiency (Figure 21). The literature suggests some increase in ROS production with age [248, 249]; however, study #2 found no impact of age on global levels of oxidative stress in female mice aged 28 months. Therefore, is the change in oxidative damage of the gastrocnemius muscle (observed in study #3) due to alterations in antioxidant protein expression?

Study #4 – Part A:

Hypothesis –

Antioxidant proteins are decreased in skeletal muscle with aging in females but not males.

Study Design –

Male and Female C57BL6 mice aged 4 months (n = 4 of each sex) and 28 months (n = 5 of each sex) were obtained from the National Institute of Health Aging Colony. Mice were anesthetized and subjected to *in vivo* electrically stimulated muscle contractions, as described in Study #1 – part A. Following the bout of *in vivo* exercise, mice were injected with Sodium Pentobarbital (100 mg/kg). Gastrocnemius muscles were excised approximately 30 minutes following the completion of the exercise bout. The remaining gastrocnemius muscle tissue, not used for the oxidative damage assay, was homogenized, as described in detail in the methods sections of Study #3- part A. Following homogenization with acid to prevent further oxidation of antioxidants, tissue homogenate was fractionated and assayed for protein expression of antioxidants, as described in the methods below.

Statistics –

Because expected variability was unknown at the time of the study, group sizes were determined based on what is commonly reported in the literature. Data were analyzed using a two-way ANOVA (age x sex). For all statistically significant interactions, a Holm-Sidak post-hoc test was implemented. All statistical calculations were performed on Sigma Stat statistical software.

Methods –

Antioxidant blots

For the western blots assessing the effects of estradiol deprivation, sex, or aging on

antioxidant proteins in muscle, cytosolic fractions of muscle homogenate prepared at a 2-mg/mL concentration with Laemelli sample buffer were thawed. Samples were heated at 95°C for 5 min prior to loading on a 10% gel. An all blue protein standards ladder and a positive control of HeLa cell lysate were loaded on all gels. Gels were run on Biorad (system info) in a 4°C cooler for 60 minutes at 150V. This time was chosen as it is long enough to achieve separation of proteins on the gel but not so long as to run small proteins off the gel. Gels were transferred to methanol soaked-PVDF membrane in a 4°C cooler overnight at 30V. Gels were chosen at random to be coomassied for remaining proteins. Coomassie indicated that all proteins had successfully transferred to the membrane.

Membranes were blocked in LiCor PBS blocking buffer for 90 minutes, prior to adding primary antibodies and tween. Membranes were incubated with primary antibodies for anti-NAPDH oxidase 4 (abcam, ab1333303), anti-superoxide dismutase 1 (abcam ab 16831), anti-superoxide dismutase 2 (abcam, ab16956), anti-catalase (abcam, ab16731), and anti-GAPDH loading control (abcam, ab9483), at concentrations recommended by manufacturers, on a rocker in a 4°C cooler overnight. The following morning, membranes were washed 4 x 15 min in PBST, then incubated with the appropriate species secondary antibody for 60 min on a rocker in a dark box at room temperature. Membranes were again washed 4 x 15 min in PBST and then imaged on a LiCor Odessey. Images were quantified using LiCor software. Quantification boxes were drawn around each sample band and the signal was normalized to the loading control, GAPDH, for that sample (Figure 22).

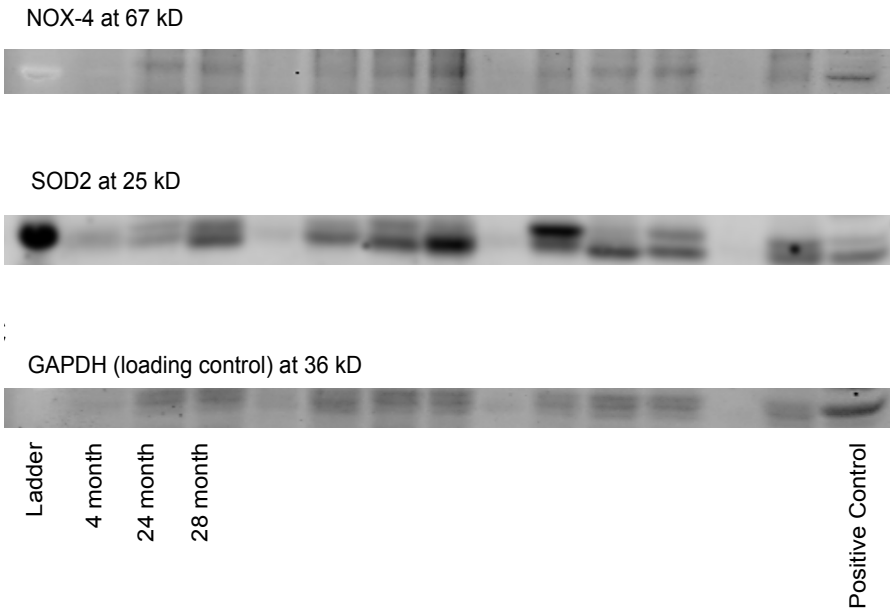

Figure 22: Representative antioxidant protein western blots showing example muscle cytosolic fraction from 4 month, 24 month, and 28 month female mice as well as positive control of HeLa cells for A) NOX4, B)SOD2, and C) GAPDH (loading control for normalization to total protein in each lane).

Blots for the antioxidant protein, GPX 3, required additional enrichment of the sample fraction due to the low abundance of the protein. For this antioxidant, a mitochondrial fraction was separated from the myofibrillar fraction. This was accomplished by further lysing cells of homogenates through an 18 gauge and then a 21 gauge needle. Sample homogenates were then spun at 1000g to bring down myofilaments and the supernatant was pipetted off and concentrated via methanol precipitation. Pellets were re-suspended in 30-50uL of PBS buffer with 6X Lamelli sample buffer. Samples were then heated, loaded and run as previously described for other western blots of antioxidants. The primary antibody utilized was anti-glutathione peroxidase 3 (abcam, ab104448).

Following quantification of GPX 3 signal, membranes were stained with Licor REVERT total protein stain and imaged for total protein content. Total protein of each lane was measured and GPX 3 signal was normalized to total protein. This was necessary because GAPDH is not expressed in the mitochondrial fraction, and common mitochondrial

loading controls are associated with the electron transport chain, which can be affected by oxidative stress.

Results –

There was an interaction between age and sex for NOX4 expression (p=0.006). Adult female mice had 11.8-fold greater expression than adult males; however, aged females had similar expression as aged males (post-hoc p<0.001, Figure 23).

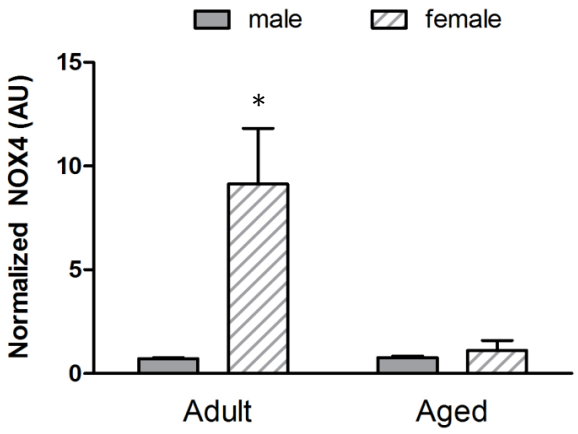

Figure 23: Aging impacts NOX4 expression differentially in the gastrocnemius muscle of male and female mice. Values are expressed as Mean ± SEM. *Indicates significantly different from adult male and aged male and female.

There was a main effect of aging on protein expression of SOD1 ($p < 0.001$). Gastrocnemius muscles from 28 month-old male and female mice had less SOD1 protein than those from 4 month-old mice. There was no effect of sex on SOD1 expression (p= 0.231, Figure 24).

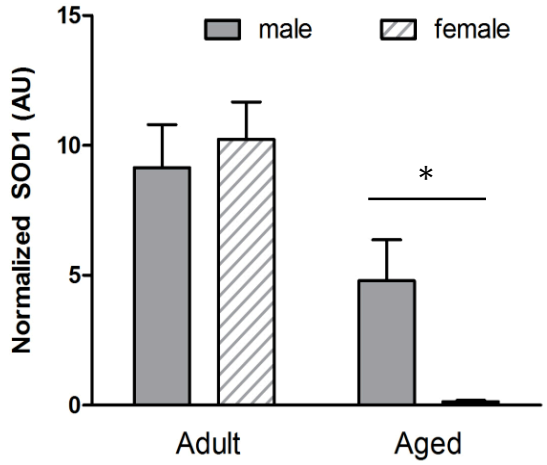

Figure 24: There is an impact of age on SOD1 protein expression in gastrocnemius muscle of male and female mice. Values are expressed as Mean ± SEM. * Indicates significantly different from same sex adult.

There was no effect of aging on protein levels of SOD2 ($p = 0.676$). There was a main effect of sex on protein expression of SOD2 ($p = 0.003$). Females have 3.2-fold greater expression than males (Figure 25).

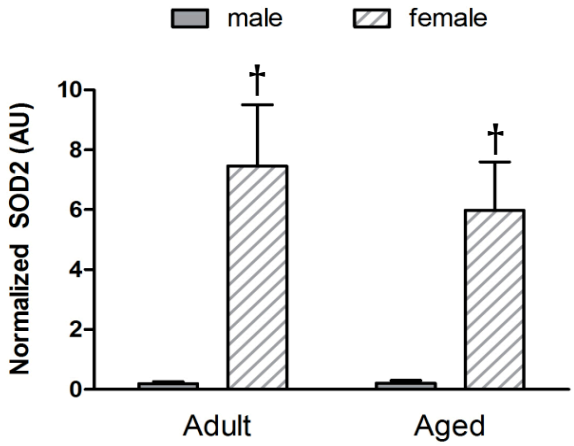

Figure 25: There was an effect of sex on protein expression of SOD2 in gastrocnemius muscle of mice. Values are expressed as Mean ± SEM. † Indicates significantly different from male at same age.

There was no effect of aging or sex on protein expression of catalase in gastrocnemius mouse muscles ($p = 0.236$ and $p = 0.399$, respectively, Figure 26).

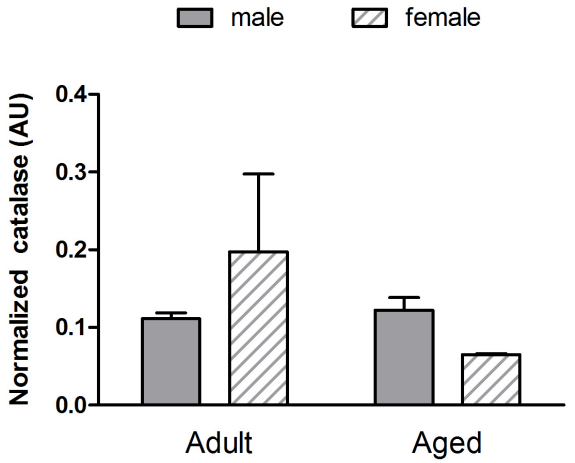

Figure 26: Catalase in gastrocnemius muscles was not different among adult and aged female and male mice. Values are expressed as Mean ± SEM.

There was an interaction between age and sex for protein expression of GPX3 ($p = 0.038$). There is no difference in expression levels between adult males and females (post-hoc $p = 0.546$); however, aged females have 1.6-fold greater expression than aged males (post-hoc $p = 0.020$, Figure 27).

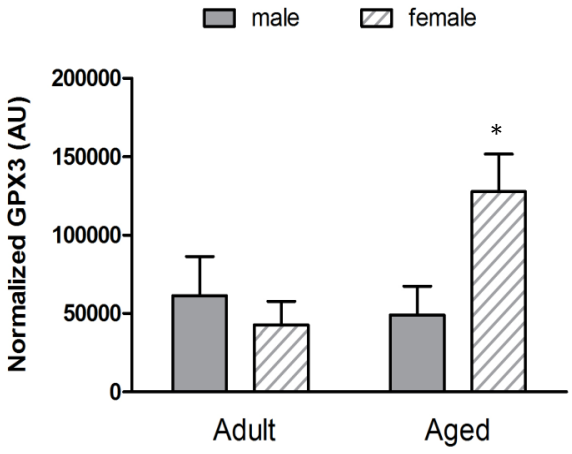

Figure 27: Aging impacts GPX3 expression differentially in the gastrocnemius muscle of male and female mice. Values are expressed as Mean ± SEM. * Indicates significantly different from adult and aged male.

Conclusions –

The impact of aging on antioxidant protein expression is antioxidant-specific, that is, some antioxidants are affected while others are not. Overall skeletal muscles from female mice appear to be more severely impacted by aging than those from male mice. Gastrocnemius muscles from aged males and females exhibit lower levels of SOD 1 expression compared to adults, and magnitude of decline is particularly large in female mice. Protein expression of NOX4 and GPX3 was affected differentially by aging in males and females. Aging did not impact expression of either protein in muscles from male mice; however, in female mice NOX4 levels were higher in aged mice whereas GPX3 levels were lower in these same mice. These results are suggestive of an aging-induced shift present only in the female mice.

The only protein to demonstrate sensitivity to sex was SOD2; female mice had higher levels of SOD2 in skeletal muscle regardless of age. Estradiol has been shown to increase SOD2 expression through estrogen receptors in the vasculature [278]; however, the results of this dissertation are the first to demonstrate an effect of sex on SOD2 protein expression in skeletal muscle. Collectively, the results provide strong evidence for an effect of aging on protein expression of antioxidants, particularly in female mice. Furthermore, for certain aspects of the SOD/Glutathione pathway, the response to aging is different in males and females. Evidence from previous work in our lab has supported a role for estradiol in antioxidant gene regulation in skeletal muscle [37]. Thus, a final study was conducted to determine the role of estradiol in modulating the expression of antioxidant proteins.

Study #4 – Part B:

Hypothesis –

Estradiol deficiency will result in decreased protein levels of antioxidants in skeletal muscle from female mice.

Study Design –

Adult and aged female C57BL6 mice were obtained from the National Institute of Health Aging Colony at 3 and 20 months of age. Mice were randomly assigned to be maintained as controls (adult n=4, aged n=4) or to undergo ovariectomy (adult n=6, aged n=6). Ovariectomy was verified by vaginal cytology. At the conclusion of the hormone deprivation, mice were anesthetized and subjected to *in vivo* electrically stimulated muscle contractions, as described in Study #1 – part A. Following the bout of *in vivo* exercise, mice were injected with Sodium Pentobarbital (100 mg/kg). Exercise was employed as a stressor to induce an antioxidant response in skeletal muscle. Baseline alterations with aging are less so impacted than response/ability to up-regulate in response to stress [263]. Furthermore, it has been suggested that the ability of tissues to respond to stressors (i.e., plasticity), is largely impacted by aging and has negative consequences for REDOX balance [282]. Approximately 30 minutes post-exercise, gastrocnemius muscles were excised, flash frozen in liquid nitrogen, and stored at -80°C. The remaining gastrocnemius muscle tissue, not used for the oxidative damage assay, was homogenized, fractionated, and assayed for protein expression of antioxidants, as described in detail in the methods sections of Study #3- part A and Study #4- part A.

Statistics –

Because expected variability was unknown at the time of the study, group sizes were determined based on what is commonly seen in the literature. Data were analyzed using a two-way ANOVA (age x ovarian status). For all statistically significant interactions, a Holm-Sidak post-hoc test was implemented. All statistical calculations were performed on Sigma Stat statistical software.

Results –

There was a main effect of aging on protein expression of NOX4 in skeletal muscle ($p<0.001$); levels were lower in aged mice compared to adult. There was no effect of ovarian status on NOX4 expression ($p=0.213$; Figure 28).

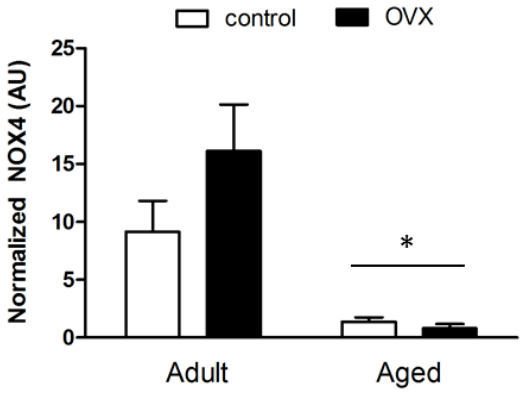

Figure 28: Aging impacts NOX4 protein expression in the gastrocnemius muscle of female mice. Values are expressed as Mean ± SEM. * Indicates significantly different from adult.

There was a main effect of age ($p=0.021$) but not ovarian status ($p=0.095$) on SOD1 expression (Figure 29). Specifically, SOD1 protein levels were 0.7-fold lower in gastrocnemius muscles of aged mice compared to adult mice.

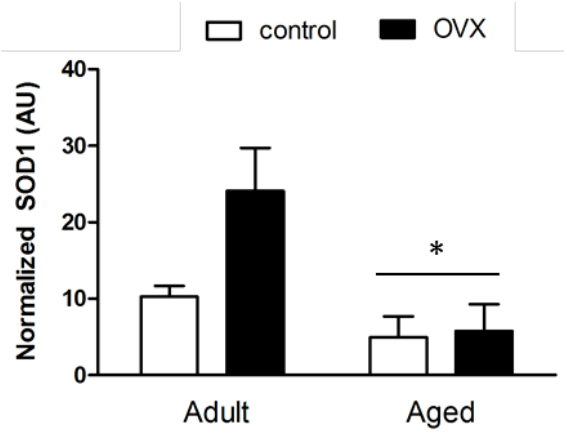

Figure 29: Aging impacts SOD1 protein expression in the gastrocnemius muscle of female mice. Values are expressed as Mean ± SEM. * Indicates significantly different from adult.

There was a main effect of age on SOD2 protein expression (p=0.018); aged mice had 0.6-fold lower levels than adult mice. There was no effect of ovarian status on SOD2 expression (p=0.183, Figure 30).

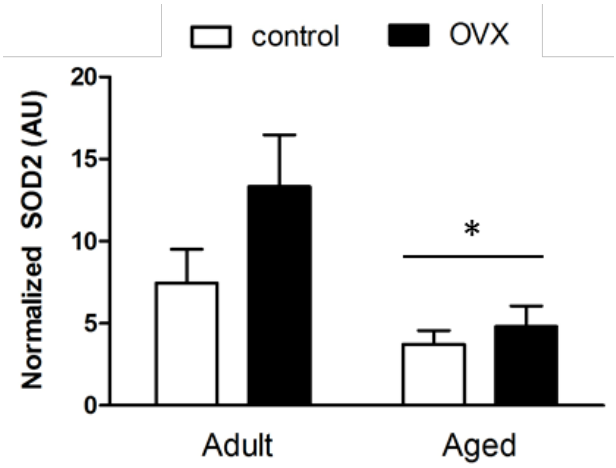

Figure 30: Aging impacts SOD2 protein expression in the gastrocnemius muscle of female mice. Values are expressed as Mean ± SEM. * Indicates significantly different from adult.

There was no effect of age (p=0.468) or ovarian status (p=0.924) on protein expression of catalase (Figure 31).

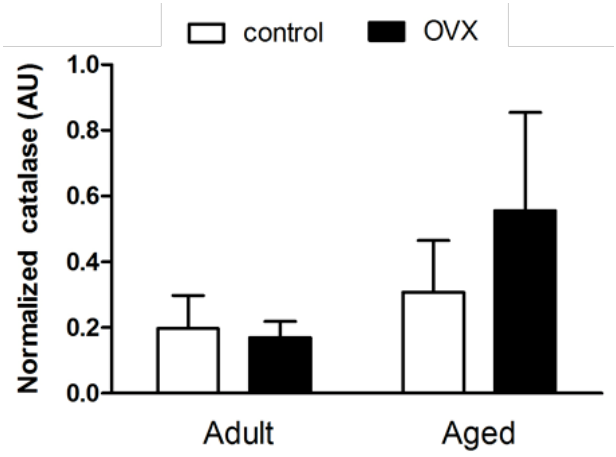

Figure 31: There is no effect of age or ovariectomy on protein expression of catalase in the gastrocnemius muscle of female mice. Values are expressed as Mean ± SEM.

There was a significant interaction between age and ovarian status on GPX3 protein expression in gastrocnemius muscles ($p=0.008$). Levels of GPX3 were higher in aged control mice compared to adult control and adult OVX mice (post-hoc $p<0.001$). Whereas ovariectomy did not impact GPX3 expression in adult mice (post-hoc $p=0.515$), removal of ovarian hormones in aged mice resulted in lower levels of expression (post-hoc $p<0.001$, Figure 32).

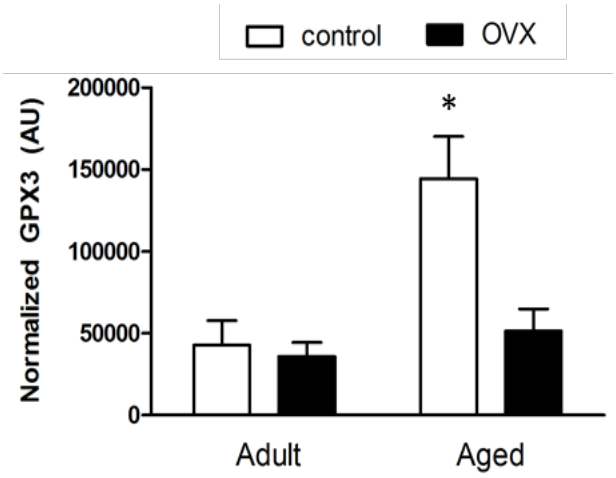

Figure 32: Ovariectomy impacts GPX3 expression differentially in the gastrocnemius muscle of adult and aged female mice. Values are expressed as Mean ± SEM. * Indicates significantly different from adult control, adult OVX, and aged OVX.

Conclusions –

There appear to be ovarian hormone-independent effects of aging on multiple antioxidants in skeletal muscle. Specifically, NOX4, SOD1 and SOD2 were lower in gastrocnemius muscle of aged female mice compared to muscle from adult female mice. The decline in antioxidant response following exercise is suggestive of a loss of redox plasticity in the muscle of aged females. To definitively show that plasticity is lost, additional control groups (muscles that were not exercised prior to excision) would need to be included. There was no impact of ovarian status on protein expression of NOX4, SOD1, or SOD2 following exercise. Although these data do not support what has been

previously shown at a gene levels, it is possible that the effects of estradiol are small compared to the impact of exercise and as such any hormone-driven alterations are overshadowed.

There was no impact of age or ovarian status on catalase protein expression, once again highlighting the importance of superoxide dismutase as key antioxidants altered by aging and loss of ovarian hormones. The significant interaction between age and ovarian status that was identified for GPX3 suggests an alteration in dismutase of superoxide into less hazardous forms or a change in the recycling of Glutathione (Figure 1). One possible explanation is that alterations upstream that occur as a result of aging (i.e., decline in levels of superoxide dismutase) may be putting additional demands on the actions of glutathione and glutathione peroxidase to neutralize additional radicals not handled by superoxide dismutase.

Chapter Discussion –

The results of study #4 demonstrate an age-associated decline in antioxidant proteins, SOD1 and NOX4, in exercised gastrocnemius muscles of female mice aged 24 and 28 months, compared to those of 6 month-old female mice. However, based on the results of studies #1 and #3, this does not appear to translate to an increase in overall muscle protein oxidation or a loss of muscle contractile function. The decline in protein expression of SOD1 with age in females is consistent with that observed in males, and is in agreement with other literature in male mice [202, 283]. Genetic removal of SOD1 has been demonstrated to induce oxidative stress and result in an accelerated aging phenotype [201], indicating the importance of this protein in mitigating oxidative stress. Unlike SOD1, the response of NOX4 protein expression to aging is sex-specific; the a decline in NOX4 expression observed in aged females was not observed with aging in muscle of male mice. This could be due to the differing levels throughout life (adulthood as well as aging) or could be indicative of an altered aging response in females and could relate to the decline of ovarian hormones following ovarian senescence in female mice. However, there did not appear to be an independent ovarian-hormone effect on protein expression of SOD1 or NOX4 in adult or aged female mice. Thus, it can be concluded that the aging effects are independent of the decline in ovarian hormone production.

The results of this study suggest a critical transition in skeletal muscle between 24 and 28 months of age in female mice. There was no impact of aging on protein levels of SOD2 in 24 month old females compared to adult; however, by 28 months of age a substantial decline in protein levels was observed. These detrimental changes are mirrored by muscle function, specifically muscle power production (e.g., loss of muscle function was not apparent until 28 months of age in study #1). These results add to our understanding of the role that SOD2 plays in maintaining muscle function, particularly with aging [128, 284].

As would be expected, based on the alterations in NOX4 and SOD1/2, protein expression of GPX3 was impacted by age in female mice. Both 24 and 28 month-old female mice had higher levels of GPX3 protein than adult females. However, there appears to be a detrimental impact of ovarian hormone deficiency on muscle from aged mice that is not observed in the adult mice. This finding gives evidence for the importance of using old mice for aging studies and not relying only on young models of aging.

Chapter #5: Conclusions and Discussion

The final chapter of this dissertation summarizes the research findings on the impact of 1) ovarian hormones and then 2) aging in regard to skeletal muscle contractile function and oxidation. The final section provides insight into possible future areas of investigation that would build upon the results of these dissertation studies.

Effects of ovarian hormones on skeletal muscle strength and power, oxidative damage, and antioxidant protein expression

As discussed in detail in chapter #1, although studies have investigated the impact of ovarian hormones (and in particular estradiol) on skeletal muscle function, there have been conflicting results not only in rodent literature but in human literature as well. Since this dissertation focused on utilizing a mouse model to investigate the impact of ovarian hormones on skeletal muscle at not only a functional level but also at a cellular level, this discussion is focused on comparisons to existing rodent literature. The meta-analysis previously published by our lab highlighted a slight overall positive impact of estradiol on muscle function in mice with some, but not all studies supporting improved force generation with estradiol [104]. In looking more closely at individual studies, the positive impact of ovarian hormones appears to be noted primarily in studies where muscle strength was measured *ex vivo* [14, 15, 18, 83, 107, 108, 285]. The decline in function was also measured at the level of myosin in skinned muscle fibers and specifically showed that the muscle quality (fraction of myosin in strong-binding state) and not quantity of contractile proteins was driving the decline in strength [15, 107, 285].

The positive impact of estradiol on muscle strength observed *ex vivo* does not completely translate to muscle contractility tested *in situ* or *in vivo*. Consistent with results of this

dissertation, previous literature also demonstrate that ovarian hormones, and in particular estradiol, do not impact maximal muscle strength [223, 286, 287]. The only positive impact found for ovarian hormones was on sub-maximal strength elicited by low stimulation frequencies [287]. As already discussed, most studies investigate the impact of ovarian hormones on maximal isometric strength of muscles; the impact on sub-maximal strength and strength produced at velocity is rarely considered. Thus, results of this dissertation add considerable insight into the impact of ovarian hormone removal on more functionally relevant measures of muscle strength.

Results from study #1 of this dissertation found minimal impact of ovarian hormones on maximal isometric torque, in agreement with the existing literature, and also minimal impact on sub-maximal isometric torque and muscle power. The effect of ovarian hormones on skeletal muscle function did not appear to be age-dependent as removal of ovarian hormones from mice at 6 or 24 months of age yielded similar results. Furthermore, longer duration of ovarian hormone deficiency, up to 12 months, did not induce additional changes in muscle function. The majority of studies investigating ovarian hormones are performed on adult mice. It is important to know that muscle of aged mice appear to respond similarly to hormone deprivation providing insight into the role of ovarian hormones across the lifespan. Thus in mice, it can be speculated that ovarian hormones are providing similar input to skeletal muscle function in adulthood and during aging.

Despite the minimal impact of ovarian hormones on muscle function *in vivo*, several key alterations were noted at a cellular level. Ovarian hormone deprivation resulted in a global decline of nitric oxide availability. Results of study #2 suggest that nitric oxide handling in the body may rely on ovarian hormone regulation. When ovarian hormones are absent, production of BH_4 (an ultimately nitric oxide) is increased, indicating a lack of nitric oxide available for tissue utilization. These results lend support for a critical role of ovarian hormones in nitric oxide regulation and are in agreement with what has been

previously reported in skeletal muscle and other tissues of the body as summarized below.

The impact of estradiol on nitric oxide production has been shown to be tissue-dependent; with estradiol resulting in up-regulation of nitric oxide in endothelial cells, immune cells, and nerve cells [288-291], but down-regulation in tissues such as the brain and liver [292-294]. In skeletal muscle, nitric oxide plays a role in muscle fatigability, fiber type determination, and post-exercise force recovery [295], as well as many other cellular aspects of muscle function and energy metabolism [296]. It has been speculated that ovarian hormones, particularly estradiol, likely impact vaso-modulation (i.e., vasoconstriction and vasodilation) of skeletal muscles during contraction [297-300]; however, current evidence is more correlative than causal.

The reasons for this tissue-disparity are not understood, but could be related to the metabolic nature of the tissue (e.g., the brain and liver are highly metabolic whereas nerves and endothelium are less so). The specific pathway of estradiol regulation on nitric oxide may occur through estrogen receptors, the expression of which has been shown to correlate with nitric oxide, specifically changes that occur with aging [301]. Based on the results of this dissertation, it seems possible that estradiol increases nitric oxide synthesis, as speculated in Figure 33. Showing cause and effect among these complicated pathways in skeletal muscle will require further investigation, as outlined in the future studies section below.

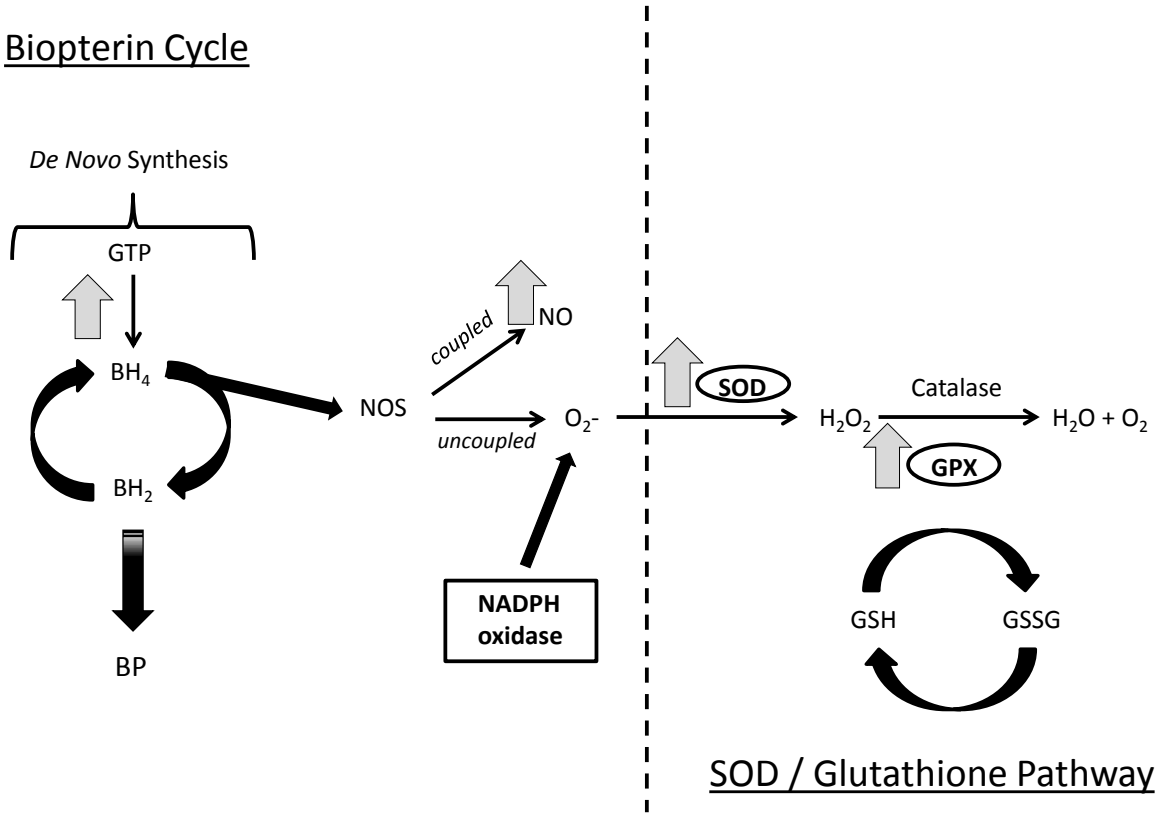

Figure 33: Speculated impact of ovarian hormones on nitric oxide synthesis and antioxidants in skeletal muscle.

Alterations in redox signaling have been implicated in age-related decline of cellular function specifically in skeletal muscle [231, 302-309] . This dissertation sought to investigate the impact of redox signaling, in particular antioxidant response, on alterations in muscle function. In order to evoke an antioxidant response, exercise was used to perturb the muscle and activate REDOX pathways.

Oxidative-induced changes in muscle could preferentially occur to contractile proteins or non-contractile proteins. Results of study #3 demonstrate an impact of sex on oxidation specifically of non-contractile muscle proteins. Although females exhibited greater levels of oxidation than males, this was not impacted by removal of ovarian hormones in females. Previous studies have suggested that ovarian hormones may be protective of oxidative damage to cardiac and skeletal muscle in females [18]. But on the whole,

evidence is severely lacking in the literature with only one study investigating the influence of sex on damage in muscle [310] , and no studies investigating the impact of sex on contractile versus non-contractile proteins within skeletal muscle. The results of this dissertation lend support for a sex difference in oxidation of non-contractile proteins that is not driven by ovarian hormones.

Contrary to non-contractile proteins, oxidation of contractile proteins in skeletal muscle does appear to be impacted by ovarian hormones. As hypothesized, removal of ovarian hormones in females resulted in increased levels of oxidation. To date no studies investigating oxidation of contractile proteins in females have been published. The existing studies in females have not differentiated between contractile and non-contractile proteins within muscle [18]. The results of this dissertation give evidence that deficiency of ovarian hormones results in the oxidation of contractile proteins in skeletal muscle. The mechanisms through which the presence of ovarian hormones confers this protective effect are relatively unknown. However, post-translational modifications of myosin have shown estradiol sensitivity [16] and it can be speculated that mechanisms may be similar.

The studies of this dissertation sought to further probe the mechanism of ovarian hormone protection against oxidation in skeletal muscle by investigating levels of antioxidant protein expression. Previous studies on the relationship between estradiol and antioxidants in skeletal muscle are limited, have largely focused on the vasculature, and have yielded mixed results [206, 311-313]. Estrogen has been shown to regulate antioxidant expression at a gene level in skeletal muscle [37] but to our knowledge, study #4 of this dissertation provides the first evidence of ovarian hormone impact on protein expression of these antioxidants in skeletal muscle. Collectively, the results support the hypothesis of this dissertation showing that females have higher levels of key antioxidant proteins (specifically SOD2 and GPX3) than males indicative of a sex-differentiated response to exercise. In addition, removal of ovarian hormones appears to be of greater detriment to antioxidant response to exercise in aged females compared to adults. Taken

together, these results lend support for both aging and ovarian hormone related impacts on antioxidant expression in skeletal muscle following exercise.

Effects of aging on skeletal muscle strength and power, oxidative damage, and antioxidant protein expression

Previous studies investigating skeletal muscle contractility in aged female mice utilized *ex vivo* approaches [17], and the current findings provide the first results for in vivo muscle functioning in aged female mice. Muscle performance in vivo includes input from the nerve and neuromuscular junction (NMJ). There are several changes to the neuromuscular junction suggested to occur with aging such as gradual loss of motor neuron-innervation [48, 314, 315], NMJ fragmentation [316],and decline of motor neuron conduction velocity [317] leading to impaired excitation-contraction coupling. Previous studies have shown that age-related declines in SOD1 may be partially responsible for the changes in the neuromuscular junction [318-320] and sarcopenia of skeletal muscle [283, 318].

The results of study #4 lend further support to declining levels of SOD1 as well as SOD2 protein expression in skeletal muscle with aging. Furthermore, in female mice the age-related decline is independent of ovarian hormone status. Due to the importance of SOD1 in muscle health, studies have suggested that removal of SOD1 would result in a failure to adapt to exercise such as that observed in old mice [201]. However, other studies have not successfully correlated antioxidant expression with muscle functional outcomes. The utilization of an *in vivo* muscle functional testing approach in study #1 prior to measurement of antioxidant proteins allowed for initial information on the relationship between antioxidant levels and muscle contractile function to be gleaned. Declines in muscle power were not observed until 28 months of age, whereas antioxidant changes were present at 24 months of age. The combined results of these dissertation studies show that an alteration in antioxidant protein expression precedes declines in muscle strength

and power and thus suggest that the loss of protection from oxidative stress in muscle may contribute to functional decline.

The impact of aging on oxidative stress and damage is also important to consider when investigating antioxidant responses due to the close relationship between stressor and response. There was a decline in oxidation of contractile proteins within muscle with aging. This is not what would be anticipated based on the existing literature [64, 65, 230, 321, 322]. However, as previously discussed, most previous studies have utilized males and not females. Combined, this suggests that oxidative damage to muscle in mice is likely sex-specific. There are limited human studies that also point towards a sex-specificity of oxidative damage in skeletal muscle, with men being more prone to damage than women [306, 323]. One plausible explanation for these dissimilarities observed between males and females with aging could be differences in nitric oxide handling. The results of study #2 are suggestive of a decrease in nitric oxide availability in aged female mice. The nitric oxide theory of aging hypothesizes that high levels of nitric oxide occur during aging and these high levels result in increased oxidative damage [324, 325].

High levels of nitric oxide, such as those that may occur with aging, drive the pathway preferentially towards increased production of oxidants and result in oxidative damage (Figure 34). Although not specifically addressed by this dissertation, there are likely alterations in nitric oxide synthase activity with aging as well which can have drastic impacts on the vasculature, in particular vasodilation kinetics, in skeletal muscle [326-328] and may also contribute to loss of muscle function [329].

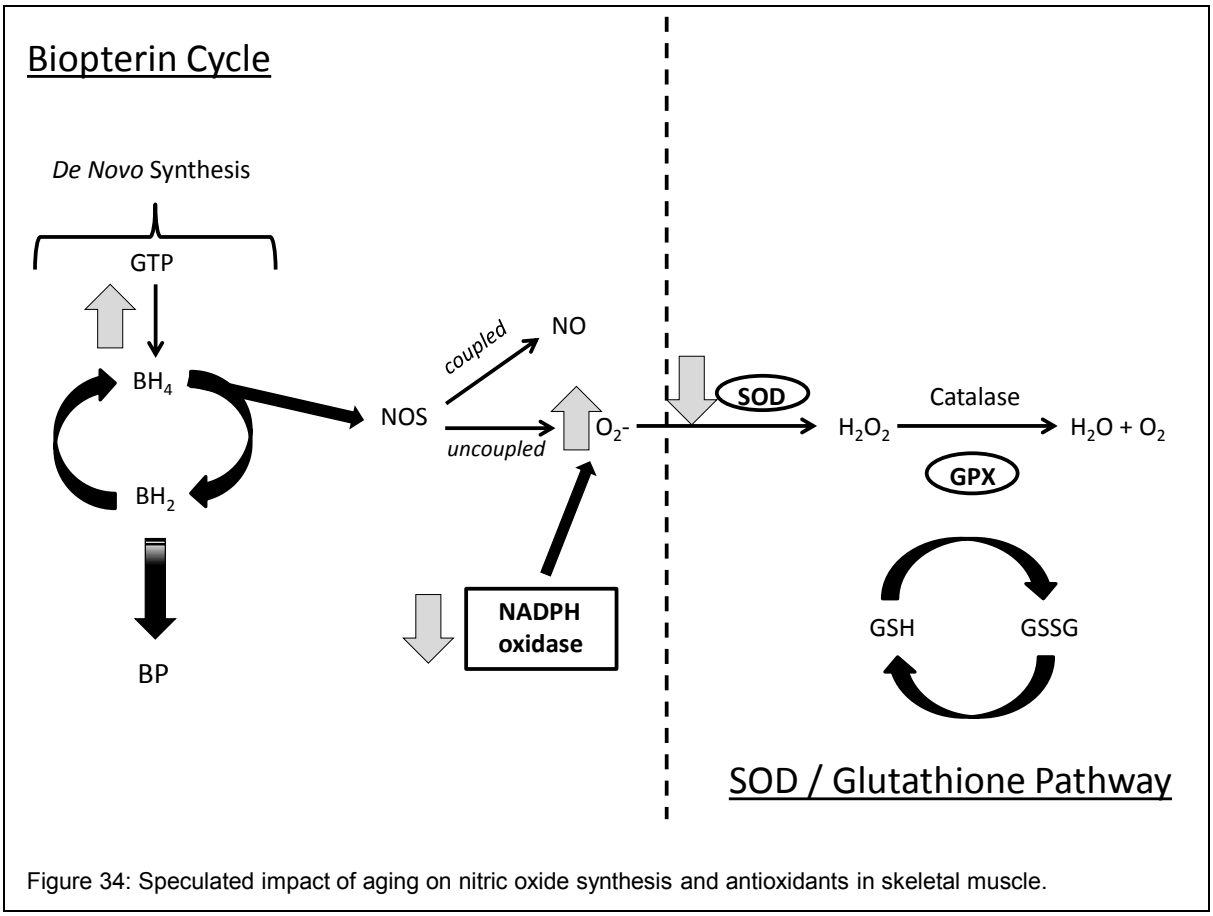

Figure 34: Speculated impact of aging on nitric oxide synthesis and antioxidants in skeletal muscle.

Overall, the collective results of these dissertation studies provide the first *in vivo* measurements of skeletal muscle sub-maximal strength and power in aged female mice. This information, coupled with the novel insights on the alterations in redox homeostasis in skeletal muscle that occur with aging, provides a potential pathway for maintenance of muscle function. Lastly, the differences observed between males and females highlight the importance of including both sexes when studying aging.

Future Directions

Based on the findings of this dissertation, further investigation into the effects of estradiol on oxidative stress in skeletal muscle is warranted. Continued work with ovariectomy in aged mice should be undertaken, since there is evidence for estradiol-independent effects as well as estradiol-dependent effects of aging. Removing ovarian tissue just prior to or at the time of senescence would remove some of the senescence-driven variability observed in the aged female mice as it would force all mice into a constant low state of estradiol. This, potentially coupled with an estradiol treatment, would strengthen the investigation of estradiol's effects with aging. Ideally, this approach would be coupled with serum estradiol measurements; however, a current limitation in the field is a lack of sensitive assays to measure circulating levels of estradiol in mice. Because the majority of commercially available assays are for human application, and mice have such low levels of estradiol compared to women (approximately $1/10^{th}$ the level), mouse serum levels are often below the level of detection.

Since the results of this dissertation clearly indicate a strong effect of estradiol and aging on superoxide dismutase and downstream antioxidants (e.g., GPX3), additional studies utilizing SOD1KO mice could provide insight to the impact of estradiol deprivation on this important pathway. This mouse model is considered to be a model of premature aging. Utilization of pharmacological approaches in this mouse could further elucidate the involvement of the estrogen receptors (ERα and ERβ) on the antioxidant response to oxidative stress. To further probe the mechanism through which estradiol may be acting, a skmERαKO mouse (mice bred to lack ERα only in the skeletal muscle) could be employed.

To further investigate the effects of aging, a mouse model of premature aging, such as the ZMP mouse, could be employed. This Progeria model would minimize the cost, time, and other limitations of aging mice to 28 months of age; however, this mouse is known to be relatively unhealthy, and as such it is possible that they may not experience regular

estrous cycles. Cytology would need to be assessed before deciding if this model would be useful to study estradiol deprivation (i.e., if the mouse is not cycling it may already be in a state of estradiol deprivation and as such it may be more appropriate to investigate the addition of estradiol in these mice).

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